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A PUBLICATION OF THE AMERICAN PHYTOPATHOLOGICAL SOCIETY

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REPORT AND ABSTRACTS OF THE 1960 MEETING OF THE POTOMAC DIVISION OF THE AMERICAN PHYTOPATHOLOGICAL SOCIETY

The seventeenth annual meeting of the Potomac Division of the American Phytopathological Society was held February 25 and 26, 1960, in the auditorium of the Plant Industry Station, Beltsville, Maryland. Thirty-eight scientific papers were presented. About 150 people attended this meeting. Following the banquet on the evening of February 25, Dr. John S. Niederhauser discussed the Plant Pathology program of the Rockefeller Foundation in Latin America and showed pictures to help him describe the scenic beauty and agriculture in Mexico.

Officers elected to serve during the next year are: President, A. B. Groves; Vice-President, J. G. Moseman; Secretary-Treasurer, R. E. Webb; and Councilor, L. O. Weaver.

JOHN G. MOSEMAN, *Secy.-Treas.*

Hemlock canker, a disease associated with poor soil drainage.—*Robert E. Baldwin and Edward S. Elliott.*—Hemlock canker is a resinous canker of nursery-grown hemlocks (*Tsuga canadensis*) of all ages. Needles of diseased trees become discolored, their lower branches die, and the basal cankers responsible for these symptoms ultimately kill the tree if they enlarge or coalesce. Cankered plants were always in poorly drained areas with water percolation rates only ¼–⅓ that of soils producing canker-free trees. Healthy hemlocks transplanted to such sites developed cankers the first year. Cankered plants transferred from poorly drained areas to well-drained areas recovered during the succeeding 1–2 years if cankers were not severe when the trees were transplanted. *Micropera abietina* was found fruiting on many cankered trees, but neither this organism nor others isolated from cankers and cankered plants produced disease symptoms on inoculation. It is concluded that hemlock canker is not of parasitic origin but is the result of abnormal plant physiology induced by poorly drained planting sites. The recommended control measure is to avoid planting sites with poor internal soil drainage.

Determination of levels of Verticillium wilt resistance in mint.—*S. Z. Berry.*—Field tests in Indiana on muck naturally infested with *Verticillium albo-atrum* demonstrated that 6 mint clones possessed a range of resistance, in order of increasing resistance: *Mentha piperita, M. spicata, M. piperita* × *M. crispa* hybrids: (148, 161, and 185), and *M. crispa*. No greenhouse technique was available for determining levels of wilt resistance between low and high. Inoculation methods were studied in a 19–26°C greenhouse, and at least 240 cuttings of each clone were used in each study. 1) Levels of resistance were expressed by cuttings after their ends had been immersed in inoculum for 48 hours and planted in steamed soil. 2) The *M. piperita* clone was least resistant and could be distinguished from the other clones when rooted cuttings were inoculated and transplanted to steamed soil. 3) Both the *M. piperita* and *M. spicata* clones could be so distinguished when inoculated cuttings were transplanted to infested soil. Tests of these three methods at soil temperatures of 10, 20, 25, and 30°C indicated that the most feasible method for determining all 6 levels of resistance was to transplant inoculated rooted cuttings to infested soil maintained at 20 or 25°C. Clones with moderate to high resistance recovered from initial symptoms. The rapidity of recovery was related to inherent level of resistance.

Growth of Cercospora kikuchii and Diaporthe phaseolorum var. sojae with amino acids and sugars in nutrient media.—*H. E. Bloss, and H. W. Crittenden.*—The incitant of purple stain of soybean seed, *Cercospora kikuchii*, and the incitant of soybean pod and stem blight, *Diaporthe phaseolorum* var. *sojae*, were grown for 10 days in a basal liquid medium. Each of the following amino acids and sugars was added to the basal medium at 5000 ppm: amino acids—asparagine, glycine, isoleucine, leucine, lysine, methionine, phenylalanine, and valine; sugars—arabinose, galactose, lactose, levulose, maltose, raffinose, rhamnose, and xylose. Mycelium was collected on tared filter papers, dried 24 hours at 40°C, and weighed on an analytical balance. For the sugars, greatest growth of *C. kikuchii* occurred with arabinose, galactose, lactose, and levulose; *D. phaseolorum* var. *sojae* grew well with all sugars except arabinose. For the amino acids, best growth of *D. phaseolorum* var. *sojae* occurred with glycine, isoleucine, and methionine whereas greatest growth of *C. kikuchii* occurred with asparagine, methionine, phenylalanine, and valine.

Cucumber mosaic virus in Nandina domestica.—*Philip Brierley and Floyd F. Smith.*—Plants of *Nandina domestica* from Maryland and Georgia bear leaflets mottled with red and green or sometimes with red blotches, rings, or oakleaf patterns. Leaflets may be normal in width and shape or fantastically deformed or reduced to midribs only. From immature Nandina leaves a virus is transmissible to tobacco by juice inoculation, and symptoms develop on the tobacco plants after 5 days in summer. Return inoculations to immature Nandina leaves by juice also succeed (13 of 29 in 16–27 days), but juice inoculations from Nandina to Nandina fail. *Myzus persicae* transmitted the virus from tobacco to 15 of 18 tobacco plants and from Nandina to 3 of 10 tobacco plants. Infected tobacco first develops ring and etch patterns and then partially recovers; subsequent leaves are smaller but normal in shape. *Nicotiana glutinosa*, petunia, tomato, cucumber, *Chenopodium amaranticolor*, *Gomphrena globosa*, snapdragon, zinnia, and cowpea are susceptible, but beans and *Dianthus barbatus* are not. Physical properties are heating 65–70°C, dilution 10^{-4}–10^{-5}, and aging 1–2 days in winter, 8–24 hours in summer. Beginning in late October, 5 of 10 infected Nandina plants grown under glass developed normal virus-free new growth.

The effect of dodine on enzymes in Saccharomyces pastorianus.—*Irwin F. Brown, Jr., and Hugh D. Sisler.*—Fermentation of glucose was strongly inhibited in cells of *Saccharomyces pastorianus* pretreated with toxic concentrations of n-dodecyl quanidine acetate (dodine). Similarly, the fermentation of glucose or of fructose, 1-6,diphosphate (HDP) was strongly inhibited in cell-free extracts from pretreated cells. Under anaerobic conditions, pyruvate was not fermented by untreated yeast cells, but cells pretreated with dodine, readily fermented this substrate. Cell-free extracts from untreated and treated yeast cells metabolized pyruvate, although activity was less in the latter extract. When HDP was the substrate, the activity of the enzyme aldolase was partially inhibited (66%) in cell-free extracts prepared from pretreated cells. No aldolase activity was detected in these extracts when glucose-6-phosphate was the substrate, although aldolase activity was present in extracts from untreated cells. HDP was not fermented by untreated cells of this organism, but when the cells were pretreated with dodine, this substrate was metabolized. The ability of treated yeast cells to metabolize exogenous substrates not commonly used by untreated cells indicates an increase in cellular permeability to these substrates. Previous work with this toxicant showed that cellular constituents are lost by cells of the yeast when they are treated with toxic concentrations of dodine. Alteration of permeability and inactivation of certain vital enzymes appear to be the mechanisms by which dodine exerts its toxic effect on *S. pastorianus*.

Wineberry, Rubus phoenicolasius, a wild host of raspberry mosaic, and an aphid vector.—*Richard H. Converse.*—*Rubus phoenicolasius,* the wineberry, occurs throughout the northeastern United States. A mosaic of wild wineberry has long been assumed to be raspberry mosaic (RM). Typical RM symptoms developed in *R. occidentalis,* black raspberry, seedlings approached-grafted to mottled wineberries. Each month from May to October, 1959, *Amphorophora rubi,* an efficient vector of the 2 component viruses of RM, was found, often in large numbers, on wineberry in Maryland and Pennsylvania. Black raspberry seedlings exposed to *A. rubi* from wineberries in the field developed typical RM. In the greenhouse, aphids (*A. rubi*) from virus-free *R. strigosus,* red raspberry, seedlings were fed on mottled wineberries collected from several locations in Maryland and Pennsylvania and were transferred to healthy black raspberry seedlings. The latter also developed typical RM. It is likely that the wineberry is an important wild host of RM, particularly when it occurs in areas where the wild red raspberry, a favorite host of *A. rubi,* is absent.

Control of Cercospora kikuchii and Diaporthe phaseolorum var. sojae on soybean seed.—*H. W. Crittenden, and H. W. Bloss.*—Six spray applications of zineb (1½ lb), maneb (1½ lb), and tribasic copper sulphate (2 lb) were made on Clark soybean at 10-day intervals from August 5 to October 1, 1958. Each fungicide gave excellent control of *Cercospora kikuchii* but no control of *Diaporthe phaseolorum* var. *sojae.* In 1959, sprays of zineb (2 lb) and tribasic copper sulphate (2 lb) were applied at 7-day intervals on Clark soybean, the number of applications and their timing being varied from July 8 to September 3. Zineb and tribasic copper sulphate applied four times from July 29 to August 19 gave almost complete control of *C. kikuchii;* one, two, and three applications of each of these two fungicides gave some control of *C. kikuchii* when timed to occur in the period July 29 to August 19. Zineb gave better control of *D. phaseolorum* var. *sojae* than did tribasic copper sulphate; six sprays of zineb from July 15 to August 19 were necessary for good control. Control of *C. kikuchii and D. phaseolorum* var. *sojae* was best when sprays were applied during the flowering period of the soybean.

Laboratory, greenhouse, and field experimentation for control of Rhizoctonia fruit rot of tomato.—*D. F. Crossan, A. L. Morehart, and W. H. Johnson.*—Rhizoctonia fruit rot occurred where several fungicides were being evaluated for tomato foliar and fruit disease control. Unsprayed rows showed 26% loss in marketable fruit. No control resulted from *N*-trichloromethylthiophthalimide (2 lb), maneb (2 lb), or a combination of both (1:1 lb). Maneb plus tribasic copper sulfate (2:3 lb) significantly reduced the loss to 11%. In greenhouse tests, these latter compounds were used alone and in combination as soil drenches. Used alone, fruit loss was 50% whereas in combination the loss was 30%. Warburg respirometric studies were conducted with Rhizoctonia mycelia grown 48 hours in shake culture and then exposed to 1000 ppm of commercial maneb or tribasic copper sulfate alone, or 500 ppm of each fungicide in combination. The reduction in oxygen uptake due to treatment was as follows: 50% from maneb, 22% from tribasic copper sulfate, 34% from maneb plus tribasic copper sulfate. The pronounced effect of maneb on respiration was not reflected in control; the relative lack of effect of copper did relate to poor control. The intermediate effect of the combination of fungicides on respiration and subsequent high degree of control suggests a type of synergistic action.

Damage to spinach and other vegetation in New Jersey from ozone and other airborne oxidants.—*Robert H. Daines, I. A. Leone, E. Brennan, and J. T. Middleton.*—A necrotic spotting of the older leaves on spinach plants was so severe throughout New Jersey in the late fall of 1958 and in 1959 that continued commercial production was threatened.

The injury first occurred as oily-appearing areas on the upper leaf surface or actually as water-soaked areas that on further development imparted a glazed appearance to the upper leaf surface. Milk-white necrotic spots in varying number occurred in the glazed area, some involving only the palisade cells and others extending through to the lower surface. A loss of chlorophyll in the upper palisade cells caused a gray-green to yellowish color on the upper surface of the oldest leaves.

Fumigation experiments with 0.25–0.30 and .40 ppm of ozone for 2–4 hours produced identical symptoms.

Ozone injury has been observed on foliage of spinach, alfalfa, rye, barley, orchard grass, tobacco, petunia, radish, red clover, bean, parsley, and grape, and perhaps chicory, endive, broccoli, carnations, and pine. On alfalfa, destruction of the chlorophyll in the palisade cells occurred at the base of the leaf. In clover and radish these injured areas were irregular in shape and location, first appearing on the upper surface and, in the radish, extending to the lower surface. Chlorophyll destruction in the cereals appeared as longitudinal streaks between the veins on both leaf surfaces. This variation in symptom expression may result from the fact that these leaves do not possess palisade cells.

Oxidant injury (silver leaf) primarily affected the undersurface of the youngest fully expanded leaves of spinach, cultivated dandelions, celery, red beets, Romaine lettuce, sour grass, petunia, snapdragon, and chickweed.

The "pecky" decay of bald cypress and a Stereum associated with it.—*Ross W. Davidson, Paul L. Lentz, and Hazel H. McKay.*—A study of cultures isolated from pecky decay of bald cypress (*Taxodium distichum*) and from a basidiocarp of a *Stereum* sp. growing on dead branch stubs of an infected tree showed that they are the same species. A study of the cypress *Stereum* and *S. sulcatum,* reported by Burt as occurring on cypress in Louisiana, revealed that they are distinct. The cypress *Stereum* is considered an undescribed species, differing from *S. sulcatum* in basidiocarp structure and in cultural characteristics. The basidiocarp of *Stereum* sp. has a grayish hymenium and prominent semi-parallel skeletal hyphae of large diameter (up to 7 μ), whereas the hymenium of *S. sulcatum* is bright salmon and the skeletal hyphae are relatively slender and inconspicuous. Cultures of *Stereum* sp. are distinguishable from those of *S. sulcatum* by their slower growth, gray color, weak or negative oxidase reaction, production of abundant chlamydospores, and lack of a conidial stage. In milled cypress lumber the common decay caused by *Stereum* sp. is known as "pecky cypress," and the infected wood is often used in paneling, in greenhouse benches, or where an antique appearance is desired. A long period of speculation as to the cause of the pecky decay ends with the discovery of *Stereum* sp.

Time-lapse studies of movement, feeding, and hatching of Pratylenchus penetrans.—*A. A. Di Edwardo.*—Photomicrographic motion pictures of adults and larvae of *Pratylenchus penetrans* in excised corn root revealed much movement of individuals within tissues. Movement in heavily infected roots appeared to be unrestricted, and apparently caused mechanical damage to such an extent that galleries were formed in the cortex. In some observations, adults were seen moving at rates exceeding 2 mm per minute. During feeding, the stylet was thrust into cells and withdrawn in rapid succession. Rate of thrust was not uniform; at times it reached 140 thrusts per minute. In the late stages of embryonic development, the young nematode could be seen coiled within the egg shell, which remained rigid until the last 48–56 hours of development. In these last hours of development, movement was frequent and vigorous and was followed by brief periods of quiescence. During this period, the stylet was thrust repeatedly against the membrane at one end of the egg. Immediately before hatching, the egg shell became thin and extremely plastic.

Hatching occurred when the young larva broke through one end of the egg shell and became completely free in a matter of seconds.

Spread of a "house-decay" fungus through soil to a standing Virginia pine tree.—*J. D. Diller, and E. J. Koch.*—In previous experiments the authors grew *Poria incrassata* under mounds of forest leaves in a wooded area at Beltsville, Maryland. During these experiments several overstory trees died. One 10-in. d.b.h. Virginia pine tree 40 ft tall broke off 3 ft from the ground during a 1959 windstorm. Examination of the wood showed diagnostic characteristics of the *Poria incrassata*. Cultures made from small cores collected from the basal 6 ft of the trunk were invariably contaminated. Three-inch sections taken at 2-ft intervals to a height of 32 ft were placed on soil in the area. Each section was covered with moist leaves. After 3 weeks, characteristic white mycelial growth appeared on the lower six sections only. The Northeastern Forest Disease Research Laboratory identified the fungus as *Poria incrassata*. This fungus apparently spread from the infestation in the original plots through the soil to the standing tree. To our knowledge this is the first convincing evidence that this fungus can attack standing timber.

Morphological variation in Tylenchorhynchus claytoni.—*E. R. Hasbrouck and W. R. Jenkins.*—Six field populations from different areas, found associated with rose, rhododendron, turf, rye and two with tobacco and six populations from a common source cultured simultaneously on tobacco, alfalfa, peas, wheat, oats and corn were sampled. Twelve morphological characters on adult females were analyzed for variability. These include the L (length), b (length ÷ esophagus), c (length ÷ tail), and V (length ÷ distance to vulva from anterior end) of de Man's formula, the linear measurements used in the ratios, stylet length, distance of the median bulb from the anterior end of the nematode, distance of the excretory pore from the anterior end, distance of the phasmid from the anus expressed as a percentage of the tail length and number of striae on the tail. Of these characters, the four involving the tail were the most variable. The V value was the least variable. The b and c values proved to be more variable than the linear measurements from which they were derived. There was no apparent host influence on character variability. Field populations varied more than did populations cultured in the greenhouse. The variability of these characters was such that, whether used independently or in combination, they proved useless for intraspecific discrimination.

Lead arsenate as a summer fungicide on apples for control of secondary scab, Botryosphaeria fruit rot, and sooty blotch.—*J. W. Heuberger, and J. J. Albert.*—In a block of Rome at Bridgeville, Del., sprayed with captan (2 lb) through second cover, the following materials were applied with a speed sprayer to 4-row plots (88 trees) from third cover (May 28) through the thirteenth cover (September 19): no fungicide (4 trees), lead arsenate + lime (1½:1½ lb), captan (1½ lb) alternating with captan + lead arsenate (1½:3 lb), captan + lead arsenate (1½:1½ lb), captan + zineb (¾:¾ lb), and captan + zineb + lead arsenate (¾:¾:1½ lb). Data obtained for the treatments showed that lead arsenate, used with lime, reduced the incidence and severity of all three diseases; when used in combination with the fungicides, it improved control. Also, it improved fruit finish and color of the foliage. At Newark, a combination of nabam + lead arsenate + lime (2.32 qt:1.7:1 lb.) was compared with captan + zineb (¾:¾) and no fungicide from third cover (June 1) through ninth cover (August 17) for sooty blotch control on 4 varieties (Jonathan, Red Delicious, Golden Delicious, Stayman). Under conditions of severe infection, the combination gave better control than captan + zineb; also, it was non-injurious to foliage and fruit except on Golden Delicious.

Morphological and nutritional differences between isolates of Gloeodes pomigena.—*K. D. Hickey and F. H. Lewis.*—Fifty isolates of *G. pomigena* from apple fruits were studied to determine whether sooty blotch is caused by one or several fungi. All isolates caused the disease when inoculated onto apple fruits of 4 varieties. The isolates showed no major morphological difference in culture, but could be separated into 4 previously described groups by observing the thallus type and cuticle cracking on the apple fruit. Each thallus type was distinct from the time of initiation and was consistently reproducible. One isolate representing each group was grown at 24°C in liquid medium containing various nutritive compounds. The isolates varied widely in the amount of dry weight produced on 21 carbon and 24 nitrogen compounds. They differed in their response to various concentrations of iron, zinc, and manganese. Each isolate had a different optimum pH level (range 5.5–6.3). The isolate producing the rimate growth pattern grew best at 20°C while the isolates producing the ramose, fuliginous, and punctate patterns had an optimum temperature of 24°C. Varieties of *G. pomigena* could be described, but no apparent useful purpose would be served.

Strain of tobacco ringspot virus found masked in sweetpotatoes.—*E. M. Hildebrand.*—A strain of tobacco ringspot virus was isolated from sweetpotatoes in 1957. During isolation studies on sweetpotato viruses from several places, distinctive systemic symptoms developed on Scarlett O'Hara morning glory from 2 sources, the Acadian and Triumph varieties. After warm storage, cork lesions developed only in the Acadian roots. The sweetpotatoes had foliage symptoms of the internal cork syndrome. When indexed the morning glory indicator initially expressed typical symptoms of internal cork, but these never became masked. Apparently the presence of the tobacco ringspot contaminant in the virus mixture made the symptoms systemic. Thermal inactivation suggested the probability of a mixture of viruses. Host-range studies demonstrated symptoms simulating tobacco ringspot on several different Nicotianas, but only when employing these two sweetpotato sources. Whether the ringspot contaminant was in mixture or alone, the morning glory indicators also exhibited severe necrotic foliage spotting. No symptoms were produced on sweetpotatoes. Aphids picked up only the internal cork syndrome from the mixtures. Host range, cross-inoculation, thermal inactivation, and serology tests established these sweetpotato ringspot isolates as being tobacco ringspot virus.

Sweetpotato and morning glory seedlings as indicators for sweetpotato viruses.—*E. M. Hildebrand.*—Graft inoculation, the basic criterion for identification of horticultural plant viruses, was the first method used at Beltsville in the search for indicator plants susceptible to sweetpotato viruses. By comparing grafting with other methods in 3 series of host-range experiments over the past 5 years, many sweetpotato and morning glory seedlings were found unsuitable for graft transmission. The chief impediments to producing good graft unions were physical and physiological (disparity in sizes of scion and stock and incompatibility of varieties and species). For the internal cork and leafspot syndromes graft transmission was largely supplanted in 1955 by the squeeze-rub rapid-inoculation technique and the aphid vector, to early establish a relatively wide host range. Failure of the grafting method made it impossible to determine the host range of feathery mottle until after the discovery of the new abutilon whitefly vector, in 1958. This vector transmits only the yellow dwarf virus component of the complex and is proving useful for extending its current very limited host range. Unless or until inoculation and growth status are synchronized to produce current-season symptoms the morning glory is not amenable to whitefly transmission. Dodder transmission was tested unsuccessfully.

Attainment of an improved level of resistance to infection by tobacco mosaic virus in tobacco.—*Francis O. Holmes.*—Crosses between T.I. 245 and other types of tobacco have yielded new breeding lines that are more resistant to infection by tobacco mosaic virus than any previously available. The new lines, though highly resistant, produce necrotic lesions in their inoculated leaves if inocula are of high titer. These primary lesions are late in developing, remain very small, and are few in comparison with those developing in similarly inoculated plants of earlier available resistant lines.

New aids for forecasting downy mildew of lima bean.—*R. A. Hyre.*—Downy mildew of lima bean (caused by *Phytophthora phaseoli*) has been forecast primarily from rainfall and temperature data. The forecasts have emphasized the first outbreak of the season. To supplement the standard method based on rainfall and temperature data, especially for later-season spread, other forecast aids were investigated. These were based on temperature and relative humidity or dew. Calculated disease-severity values varied with the presence of dew or a relative humidity of 90% or more for 10 to over 25 hours, and depended on a concurrent mean temperature between 45 and 80°F and on the maximum temperature the following day. Positive correlation with disease spread was better when the limiting maximum temperature the following day was 85° rather than 90 or 95°. The same relation held for "Beaumont" infection periods. These were considered to have occurred when the relative humidity was 75% or more for 48 consecutive hours or more. In 1959 the new aids verified the standard method but were no improvement over it.

The effect of maneb on the oxygen uptake of Colletotrichum capsici in the presence of substrates found in oxidative pathways.—*W. H. Johnson and D. F. Crossan.*—The mycelium from spores of *C. capsici* was grown for specific periods in shake cultures containing a basal mineral medium with a single carbon source. The substrates used were intermediates or starting products of glycolysis, the citric acid cycle, or the hexomonophosphate shunt. At the end of a set time for each substrate, the oxygen uptake was measured on a Warburg apparatus using the mycelium with and without 1000 ppm of commercial maneb (70%) for 80 minutes. A statistical analysis of the data indicated no difference between manometers within a given treatment and significant differences between specific substrates. The difference in per cent oxygen uptake of the maneb-treated mycelia for each substrate, expressed as a minus or plus value in relation to the untreated mycelia, was as follows: galactose, none; fructose, none; glucose, −50%; mannose, −56%; pyruvate, none; citrate, none; succinate, −43%; and, fumerate, +37%. This indicates a block between succinate and fumerate, and a possible interruption in phosphorylation of glucose and mannose.

Symptoms of Verticillium wilt in yellowpoplar.—*K. J. Kessler and R. P. True.*—Symptoms due to *Verticillium dahliae* appeared in the single tree studied in June, 1958, with a drooping and yellowing of leaves on a few lower branches. Leaves on some branches wilted rapidly and turned brown, and the buds died during the summer. On other branches, marginal and intercostal blackening gradually involved most of the leaves, and defoliation followed without immediate killing of buds. By the fall of 1958, all branches showed some symptoms and many were dead, though some still showed chiefly droop and yellowing. During the winter, additional branches died and limbs refoliating in the spring of 1959 produced dwarf leaves and, in July, abnormally small flowers. In August a second crop of small flowers appeared. A few water sprouts developed during 1959. In August, after the second flowering, the dwarf leaves showed marginal browning, more branches died, and the tree was cut. Extensive purple vascular discoloration was found in the trunk, roots, and larger limbs but not in the small twigs. The fungus was often but inconsistently isolated from the discolored wood.

Evaluation of 211 varieties and 3 species of gladiolus for resistance to Fusarium oxysporum f. gladioli in 1959. *John G. Palmer and R. L. Pryor.*—Corms of 173 varieties and 3 species of gladiolus not tested in 1958 were inoculated by rotation on a wire grid in sand saturated with a composite inoculum of 6 isolates and were planted in gravel in subirrigated benches. Twelve varieties plus 1 species were resistant enough for use in studies of inheritance of disease resistance. Eighty to 100% of the inoculated corms of 52 varieties and 1 species rotted before germination. For the 38 varieties screened in both 1958 and 1959, the spans for development of yellows in 100% of shoots from inoculated corms were similar but were delayed up to 2 weeks in 1959. Use of smaller corms freer of disease, addition of browning of the outer sheath as a requirement for marking a shoot symptomatic, and cooler temperatures in 1959 caused this delay. In a few varieties, number-5 corms rotted whereas jumbo corms germinated. Some shoots from jumbo corms of two of these varieties were asymptomatic for yellows after 14 weeks. Corms of equal size from different geographic sources reacted similarly. *Gladiolus dracocephalus* and *G. primulinus* are grown commercially. The latter was classed resistant. All inoculated corms of *G. byzantinus* rotted, but controls neither rotted nor germinated. Sample corms of all species and varieties grew when planted in soil.

Relative susceptibilities to blackspot of some rose varieties in excised leaflet assay during 1959.—*John G. Palmer and Peter Semeniuk.*—A modified detached-leaflet technique was used in 13 inoculations between May and November. A leaflet from each of 3 leaves (first fully expanded) was inoculated with 1 drop from a 26-gauge needle containing 2½ million spores per cc of *Diplocarpon rosae* obtained from field-grown bushes of Red Radiance. Leaflets were kept at 72°F and were examined at 20× magnification 7–10 days and 13–17 days after inoculation. The recorded steps in infection were subcuticular hyphal strands; strands plus blackening; acervuli from which typical, two-celled spores were extracted; and acervuli with spores plus blackening—respectively assigned the values 1, 2, 3, and 4. No evidence of infection was given 0. An index average for the inoculations was obtained by dividing the total points by the total number of leaflets inoculated. Ratings of resistance based on this index match general field ratings more closely than do ratings on a + or − basis. Of the 9 hybrid teas (HT), 2 hybrid perpetuals (HP), 2 floribundas (F), 1 long rambler, 1 tea, and 1 climber assayed, Goldilocks (F), and Chic (F) were very resistant; Georg Arends (HT), Black Prince (HP), Peace (HT), and Red Radiance (HT) were moderately susceptible in sequence. Of the most susceptible, 3 were hybrid teas and 2 were seedlings from selfs of the floribunda Ma Perkins.

Effects of phaltan and cycloheximide applied separately and in combination upon powdery mildew and plants of blackspot-resistant Rosa wichuriana.—*John G. Palmer, P. Semeniuk, and T. J. Henneberry.*—Three rows of seedlings from open-pollinated seed of *Rosa wichuriana* were sprayed at high dosages for 6 months during 1959; alternating rows were not. Phaltan without a spreader markedly reduced infection of new growth but did not eradicate mildew. Within 72 hours of the initial applications in May, the plants sprayed with Actidione PM were free of powdery mildew mycelium, the red color in young stems and buds had deepened, and leaves and stems were stiff. Within 2 weeks, foliage that was symptomatic at the time of the initial applications containing actidione had become red-spotted and yellowed. Severe heat and constant wind during late June removed old, infected leaves from unsprayed plants, and a nearly equal number of leaves abscissed from plants sprayed with actidione. Foliage of

plants sprayed with phaltan was a dark green, with actidione a light green, with the combination yellow-green. In mid-October observers selected the sprayed rows as more foliate than the unsprayed, with total growth decreasing in the following order for actidione, phaltan, phaltan plus actidione, and none (checks). Actidione stimulated axillary-bud initiation, and growth of these initials accounted for the larger plants. When combined with phaltan it stunted this growth.

The nutritional competition between Hypoxylon punctualatum and Ceratocystis fagacearum.—*R. W. Roncadori and R. P. True.*—The cellulolytic fungus *Hypoxylon punctulatum* is often found causing a rapid sapwood decay in deep-girdled oak wilt trees. This fungus was used in an experimental control study to determine whether it would limit longevity and sporulation of *Ceratocystis fagacearum*. Inoculation of *H. punctulatum* into fresh deep-girdle wounds increased its incidence and distribution but did not reduce the frequency of isolation of *C. fagacearum* after 10–12 weeks. The oak-wilt fungus grew poorly on a medium made of bark or sapwood decayed by *H. punctulatum*. Supplementing the above medium with a suitable nitrogen source resulted in good growth of *C. fagacearum*. Nitrogen was demonstrated in decayed sapwood by chemical analyses but appeared to be unavailable to the oak-wilt fungus. It is concluded that rapid growth of *H. punctulatum* and the development of its sapwood decay lead to nutritional conditions unfavorable for continued growth and sporulation of *C. fagacearum*.

Problems with germination of oospores of Phytophthora infestans.—*E. J. Savage, and M. E. Gallegly.*—Oospores of *Phytophthora infestans* produced in paired cultures, germinated in horse dung infusion, potato soil leachate, coumarin, 2,4-dichlorophenoxyacetic acid, gallic acid, 3-indolepropionic acid, caffeine, and plates of germinating potato and tomato seeds. The highest percentage germination obtained with these treatments was 12.5 with coumarin. Two oospores kept at 10°C. under water, germinated after 9 months. Oospores treated with 2,4-D and then irradiated with ultraviolet light for intervals of 30 seconds to 32 minutes showed a maximum germination of 12.5% (in one plate) and an over-all germination of 3.5%. Oospores, treated with ultraviolet light alone, showed a maximum germination of 6.0 per cent. Many physical and chemical treatments failed to increase the percentage germination. All oospores germinated by producing a germ-tube or a germ-tube terminated by a sporangium. Two single-oospore cultures were established. Both were found to be physiologic race *1,2* and compatibility type A_1. Genetic recombination was indicated since both were derived from pairing a Mexican isolate of physiologic race *1,2,4* and compatibility type A_2 with a Puerto Rican isolate of physiologic race *3* and compatibility type A_1.

The utilization of $C^{14}H$-COOH by non-germinated spores from fungi of varying parasitic ability.—*Richard C. Staples and Harry P. Burchfield.*—The utilization of the methyl carbon of $C^{14}H_3COOH$ over a 24-hour period by greenhouse-grown uredospores of *Uromyces phaseoli*, *Puccinia graminis* f. sp. *tritici* race 56, and *Puccinia helianthi* was compared with that of conidia from PDA cultures of *Glomerella cingulata*, *Neurospora sitophila*, *Aspergillus niger*, and a field collection of chlamydospores from *Ustilago maydis*. Following extraction of the spores with 80% ethanol and hot water, C^{14} determinations were made of the amino acids, organic acids, non-ionic materials, proteins, nucleic acids and the residue using ion-exchange resins and paper chromatography. Uredospores utilized only 5% of the acetate that the less-obligate spores did as judged by their C^{14} contents. Proportionately more activity was found in the protein fraction from some non-obligate spores than in uredospores, but these differences were small in the case of *A. niger* conidia and corn smut chlamydospores.

When the distribution of C^{14} among the free amino and protein amino acids was studied, most protein amino acids from spores of *N. sitophila*, *U. maydis*, and *A. niger* were found to be rapidly labeled while they became labeled only slowly in uredospores. Even after 24 hours the leucines and valine, among others, did not become labeled although they were labeled in the free amino acid fraction by 10 hours.

Observation of plant-parasitic nematodes on the roots of living plants using a glass-sided root observation box.—*H. T. Streu.*—Distribution and feeding of *Criconemoides curvatum* on roots of living carnation plants was observed through a stereoscopic microscope set directly upon the glass sides of a root observation box. This box was constructed from 1×2-in. spruce grooved for the reception of common window glass. The resulting chamber, 1 ft square and 1 in. thick, was filled with autoclaved soil and planted with a nematode-free rooted cutting. Specimens of *C. curvatum* (250) in water were poured over the upper surface of the plant roots. Feeding areas were observed and recorded periodically and root feeding sites were recorded with photomicrographs. Nematodes remained more concentrated near the area of inoculation and became slowly distributed throughout the soil. Migration was primarily vertical from the point of inoculation. Young larvae were the most active forms, whereas mature individuals moved the least. Flush of water through the soil is primarily vertical and may be the vehicle of distribution of this species. Active feeding was observed on root tips, on the root hair zone, and on older roots. Population densities were greatest on the root hair zone, however.

Fungicide-acaricide combinations for the control of Diplocarpon rosae.—*R. V. Travis and T. J. Henneberry.*—Fungicides—70% maneb, 76% ferbam, 65% zineb, 50% captan—were combined with acaricides—15% Aramite, 25% malathion, 26.2% demeton, 47.5% phorate (Thimet)—to test their efficiency for the control of *Diplocarpon rosae* and *Tetranychus telarius*. These materials were applied at the rate of 2 lb of wettable powder per 100 gal. of water except demeton, 13.4 fl. oz., and phorate, 6.4 fl. oz., which were used as emulsifiable concentrates. All sprays also contained 2 lb of 50 % DDT, 1 lb of 25% lindane, and ½ pint of Santomerse S per 100 gal. These sprays were applied weekly or biweekly (demeton, phorate) from June 18 through Sept. 17, 1957. About ¾ gal. of spray was applied to 24 rose plants in each treatment. The Helen Traubel rose was inoculated with a spore suspension of *D. rosae* on Aug. 7 and rated for severity of infection on Aug. 27 and Oct. 4. Analysis of the black-spot ratings (possible range of 0–800) showed maneb (164) and zineb (182) superior to ferbam and captan, ferbam (253) better than captan (316), and captan better than no fungicide (688). Aramite (290) and demeton (297) rated better than malathion (330) and phorate (329) in fungicidal sprays, and all were significantly better than no acaracides (356). No difference in yield of flowers was evident between any of the treatments.

Resistance to stem anthracnose of lima beans.—*R. E. Wester.*—Stem anthracnose, caused by *Colletotrichum truncatum*, has a wide distribution, especially in the Southeastern States, where it occurs on lima beans and occasionally on snap beans. Its known distribution includes Alabama, Georgia, Louisiana, Mississippi, North Carolina, Pennsylvania, South Carolina, Texas, Virginia, Maryland, and Iowa. In 1959 this disease was so severe in Delaware and on Eastern Shore, Maryland, that yields were reduced by 50–75% in commercial fields where no fungicides were used. The disease also occurred in New Jersey in 1959, but was not severe enough to affect lima bean yields.

All commercial varieties and most of the Foreign Plant Introductions tested were very susceptible to the disease. In the fall of 1959, however, two resistant Introductions were found—P.I. 199,791 from British Guiana and P.I. 186,984 from South Africa. Both have vigorous vines. The

one from South Africa has large, flat white seed. That from British Guiana has large, flat seed that are burnt-tan and black mottled with a black eye. Its seedlings are especially vigorous and have purple hypocotyls. These anthracnose-resistant lines are to be crossed with Fordhook 242, Thaxter, and other commercial varieties.

Nutrition and environment in relation to development of late blight on resistant and susceptible varieties of tomatoes.—*J. B. Wilson, and M. E. Gallegly.*—The relation of nutrition and environment to disease development was studied on 5 tomato varieties and accessions: 1 susceptible variety, 2 multigenic resistant, and 2 dominant-gene resistant accessions. Susceptibility increased as the balanced nutrient concentration was increased from 0.1H to 3H. The multigenic varieties displayed resistance at the 0.1H level. In unbalanced nutrition tests the multigenic accession 19 displayed resistance at the low N level. The multigenic resistance of accession 19 was broken down under postinoculation low light when inoculated with tomato race 0.

Both multigenic accessions displayed resistance under postinoculation high light when inoculated with tomato race 1, even when predisposed to low light. Postinoculation low light increased susceptibility in all tests. Accession 19 displayed resistance to tomato race 1 under long daylength. Accession 19 resistance was broken down at 16°C air temperature but held up at 24°C when inoculated with tomato race 0. Dominant-gene accession 36 was the only accession displaying resistance to tomato race 1 when postinoculation temperature of 24°C was maintained regardless of pre-inoculation temperatures. All varieties and accessions become more resistant with age from 7 to 39 days from transplanting.

A new disease-resistant Great Northern bean.—*W. J. Zaumeyer, H. Rex Thomas, and M. M. Afanasiev.*—Great Northern 1140, a new early, multiple-disease-resistant dry bean, is now available to growers in areas where Great Northern type beans are grown. Its parents are Great Northern U.I. 123, a mosaic-resistant variety, and U.S. 5 Pinto, a rust-resistant type. Great Northern 1140 is immune to two virus diseases—common bean mosaic and the New York 15 strain of this disease. It is also resistant to many of the 30 reported races of bean rust. Because of its growth characteristics, allowing for more air circulation near the base of the plants and within and between the rows, it is likely to escape damage from sclerotinia wilt, common to heavily foliated varieties. Its performance records show that it is widely adapted to the bean-growing areas of Nebraska, Wyoming, Montana and Idaho. Under commercial conditions in Idaho in 1958 and 1959, Great Northern 1140 outyielded the other Great Northern varieties by about 350 to 500 lb/A (average 15–20%). The largest yield for the new variety was 62 bu/A. About 4½ million lb of certified seed was produced in Idaho in 1959. It is being released jointly by the U. S. Department of Agriculture and the Montana and Idaho Agricultural Experiment Stations.

REPORT AND ABSTRACTS OF THE 1960 ANNUAL MEETING OF THE SOUTHERN DIVISION OF THE AMERICAN PHYTOPATHOLOGICAL SOCIETY

The Southern Division of the American Phytopathological Society held its annual meeting in conjunction with the 57th annual convention of the Association of Southern Agricultural Workers, at Birmingham, Alabama, February 1–3, 1960. About 130 plant pathologists attended; 24 papers were presented. The afternoon of February 1 was devoted to a symposium on *Sclerotium rolfsii*. Papers presented at this symposium will appear in a later issue of *Phytopathology*. At the business meeting, February 1, it was announced that the following officers had been elected by mail ballot: *President*, W. J. Martin; *Vice-President*, A. L. Harrison. W. W. Hare continues as Councilor.

J. P. FULTON, *Secretary-Treasurer*

The influence of preplanting treatments of root-knot-infested gladiolus corms and row applications of nematocides on nematode control and production of corms and flowers.—*Robert Aycock.*—When corms infested with root knot (*Meloidogyne incognita acrita*) were treated 4 hr. at 43°C in 0.5% formalin, no nematodes survived. Treatment with New Improved Ceresan (2 lb/100 gal. water for 15 min.) or Dowicide B (3 lb/100 gal. water for 15 min.) was ineffective in nematode control, and Dowicide B caused injury and reduced yields. Treatment of corms in hot water at 50°C for 10 min. did not eradicate root knot nematodes but stimulated plant emergence and subsequent vigor. Yields and quality of flowers from infested corms planted in soil treated with Nemagon (0.5 gal. technical/acre) or EN 18,133 (2 gal. technical/acre) in the row equaled those from corms that also received a preplanting eradicative treatment. These responses were correlated with low nematode populations at the end of flowering. Yield of Number 1 and Jumbo corms was greatest and of highest quality from infested corms treated 4 hr. in 0.5% formalin at 43°C and planted in Vapam-treated soil. At harvest, root knot nematode populations were 3-6 times as great in Vapam-treated plots planted with infested corms as when such corms were planted in untreated soil.

Effectiveness of certain protectant fungicides for control of pecan scale in Oklahoma during 1959.—*George Barnes.*—Protectant fungicides were sprayed 10 times onto Western pecan trees following a 2-week schedule for control of the pecan scab fungus, *Fusicladium effusum*. The inoculum level during the season was very high, rains were frequent, and unsprayed trees became severely scabbed. Zineb fungicides (Parzate, 3-100; Dithane Z-78, 3-100; and zineb tank mix); Cyprex, 1-100; and Dyrene, 4-100, provided excellent control, low-lime Bordeaux Mixture (6-2-100) provided good control; but ziram-based materials (Zerlate, 3-100; Niacide Z, 4-100, and ziram tank mix); Amobam, 1 qt.-100; and Dibam A, 1 qt.-100, gave very poor control. The average number of nuts per lb. harvested from the treatments were: Parzate, 58; Parzate plus summer oil, 67; Dithane Z-78, 57; zineb tank mix, 74; Cyprex, 84; Cyprex plus summer oil, 62; Dyrene, 64; Bordeaux, 78; Zerlate, 112; Niacide Z, 124; ziram tank mix, 93; Amobam, 108; Dibam A, 146; and untreated, 215.

Techniques for evaluating sweet potato lines for their reaction to soil rot.—*Lester W. Carlson and F. Ben Struble.*—Before inoculation tests could be made to determine the reaction of sweet potato lines to *Streptomyces ipomoea*, the casual agent of soil rot, it was necessary to devise a reliable technique for testing pathogenicity of *S. ipomoea* isolates. The most satisfactory method involved the use of pieces of freshly dug susceptible sweet potato roots 3-6 mm in diameter. Following inoculation these pieces were embedded in slightly moistened sterilized vermiculite in 145×20-mm Petri dishes and incubated at 32°C for 5-7 days. No technique involving artificial inoculation was found satisfactory for distinguishing varietal reaction to soil rot in sweet potato. Varietal resistance

was greatest in lines having fewer number of layers of non-nucleated periderm cells and an abundant, rapidly developing root system.

Summer killing of strawberry plants caused by Colletotrichum fragariae.—*R. G. Carver and N. L. Horn.*—*Colletotrichum fragariae* was consistently associated with dying strawberry plants in beds during the summer. Field plants were also killed by the fungus in late spring when day temperatures were 80°F or higher. The fungus attacked petioles, runners, and crowns of plants. When the crowns became infected the plants died. From field observations and greenhouse inoculation tests the Headliner variety appeared more resistant to the disease than some standard Louisiana varieties. To study possible chemical control measures plants were grown in field soils treated with captan, methyl bromide, ethylene dibromide, 1,2-dibromo-3-chloropropane, pentachloronitrobenzene, and sodium *N*-methyldithiocarbamate. Highly significant differences in plant production occurred in soils treated with methyl bromide as compared to untreated soils. Parasitic nematodes were isolated from soils in 1 test, but no correlation was found between number of plants produced and the presence of these nematodes. Physiologically and morphologically, the Louisiana isolate appeared identical to the type culture.

Identification and preplant control of parasitic nematodes attacking peach trees in South Carolina. *H. H. Foster.*—Root-knot nematodes have been recognized as major pests of South Carolina peach trees for many years. The following species have been tentatively identified: *Meloidogyne javanica, M. incognita, M. incognita* var. *acrita,* and *M. arenaria.* The following genera of parasitic nematodes were frequently found associated with peach roots: *Pratylenchus, Xiphinema, Criconemoides, Trichodorus,* and *Tylenchorhynchus.* Preplant nematocidal studies were begun at the Sandhill Experiment Station in 1956. Nine different nematocidal treatments were used, each at 2 concentrations. After the first growing season, significant increase in both shoot length and trunk circumference of trees on treated plots, in comparison with trees on untreated plots, was limited to 3 nematocides: dibromochloropropane, ethylene dibromide, and methyl bromide. In 1958, the second growing season, all nematocides used, at least at one rate, except VC-13, showed a significant increase for tree height, shoot length, and trunk circumference. Root-knot galling was significantly reduced by all nematocides during both years. All nematocides tested, except VC-13, tended to prevent the early stunting of trees normally caused by root-knot nematodes.

Effects of temperature on cottony blight of ryegrass.—*T. E. Freeman.*—Cottony blight of ryegrass (*Pythium aphanidermatum*) is favored by warm temperatures with maximum severity at 35°C. Below 20°C, disease severity is negligible. Despite this affinity for warm temperatures, the disease frequently causes severe damage during Florida winters, when the mean daily temperature is below the favorable range. Thus, it appears that disease development is rapid enough to cause damage even if only a few hours of favorable temperatures occurred. To determine if this was the case, inoculated grass was exposed to 25°, 30°, and 35°C for 2, 4, 8, and 24 hours for 3 successive days. Some damage occurred even at the lowest temperature for the shortest period. Each increase in number of hours exposed and/or temperature increase resulted in more severe disease. The period required for 50% of maximum damage was: 8–24 hours at 25°C, 4–8 hours at 30°C, and 2-4 hours at 35°C.

A chlorosis of cotton seedlings caused by Alternaria sp.—*N. D. Fulton, Katharina Bollenbacher and B. J. Moore.*—When fungi isolated from diseased cotton seedlings were tested in pure culture for pathogenicity to cotton in the greenhouse, an *Alternaria* sp. isolate produced a chlorosis on the cotyledonary leaves of the seedlings. In subsequent tests 9 of 14 *Alternaria* isolates were shown to produce the chlorosis. Such chlorotic seedlings have been observed in the field, and an *Alternaria* sp. isolated from them. Cotton seed planted in either sterile sand or soil mixed with 4-week-old *Alternaria* inoculum grown in a modified Richard's solution produced a high percentage of chlorotic seedlings. Autoclaved inoculum and sterile culture filtrate of the pathogen both caused chlorosis, which indicates that the fungus produces a toxin that causes the chlorosis. Preliminary tests show that cotton variety does not influence the condition materially nor do temperature and light intensity, although low light and low temperature do retard plant growth so that the chlorotic condition is prolonged. When a sterile culture filtrate of the fungus was introduced into a lower petiole of older plants, unilateral wilting and eventual necrosis resulted. Chlorotic seedlings with affected areas greater than 35% usually die, and seedlings with less chlorosis are considerably less vigorous than non-chlorotic plants.

Comparative development of stem rot in a Virginia Bunch and a Virginia Runner variety of peanuts.—*Kenneth H. Garren.*—The concept persists that runner varieties of peanuts are resistant to stem rot incited by *Sclerotium rolfsii.* For 2 years at Holland, Va., the development of stem rot in 2 selections, Virginia Runner 56-R and Virginia Bunch 46-2, was compared. These varieties were grown in an experiment that compared 2 methods of weed control superimposed on 2 methods of seed-bed preparation. The varieties responded similarly to the treatments. Lowest infection and highest yield were always obtained from the same treatment. In 1958, at an intermediate infection level, there was more infection in the bunch variety given the 2 poorer treatments than in the runner variety given these treatments, but reduction in yield of the runner variety was proportionally greater. In 1959 only the poorest treatment resulted in obviously greater infection in the bunch variety, but this difference was not statistically significant and the effects of treatments on yield were much the same as in 1958. Virginia Runner 56-R variety is not consistently less susceptible to *S. rolfsii* than is Virginia Bunch 46-2; therefore it can hardly be considered resistant.

Reaction of 8 sweet potato selections to 5 species of root-knot nematodes.—*Mike J. Giamalva, W. J. Martin and Teme P. Hernandez.*—Sweet potato varieties Porto Rico, Goldrush, Acadian, Heartogold, Allgold, and Nemagold, and Louisiana seedlings 1-80 and 3-77 were grown in replicated greenhouse experiments in steam-sterilized soil artificially infested with *Meloidogyne arenaria, M. hapla, M. incognita, M. incognita acrita,* and *M. javanica.* Ten weeks after planting, roots of each plant were rated for galling and necrosis. The roots were weighed and the number of larvae in the soil was determined. All nematode species caused galling and root necrosis and developed to maturity on certain selections. However, selections differed greatly in their reaction to given nematode species. *M. incognita* caused galling (trace) and developed mature females in Heartogold only. On Nemagold, trace galling and development of mature females was induced by all species except *M. arenaria. M. arenaria* caused slight to moderate root necrosis on all varieties. *M. incognita* and *M. incognita acrita* caused the most severe galling on all selections. There was no correlation between galling and root necrosis. There was a significant positive correlation between root-knot gall index and number of larvae. There was no significant difference in root weights between species on any variety, and no correlation between root weights and other indexing methods.

A root-knot-resistant tobacco breeding line released to breeders.—*T. W. Graham.*—A new flue-cured tobacco breeding line, PD 611, resistant to root knot, was released in 1959 to tobacco breeders for further variety development. It has a stabilized and high level of resistance to *Meliodogyne incognita* var. *acrita,* the common root knot species on tobacco in the southeast. Growth characteristics closely resemble flue-cured varieties having good yields,

curability, and early growth vigor. The cured leaf, however, is below top quality and not acceptable to some manufacturers. Nematode resistance was derived from crosses involving the alloploid *Nicotiana sylvestris* × *N. tomentosiformis* and a breeding line from the South American introduction T.I.706. After a series of further crosses of the above to various varieties, a breeding line, Bel 4-30, was produced. Original crosses to produce Bel 4-30 were made by E. E. Clayton. Development of PD 611 followed a cross of Bel 4-30 × Hicks, the progeny of which where then backcrossed to a breeding line Bel 5-32, a line related to Bel 4-30. A second backcross was then made to Vg 2, which in turn came from a cross of the variety 402 × Virginia Gold. After a series of 8 selfed generations, PD 611 proved to be stable in nematode resistance and was uniform in field appearance.

Thiram for azalea petal blight control.—*Clinton H. Graves, Jr. and T. E. Ashley.*—Outstanding results were obtained for control of azalea petal blight (*Ovulinia azaleae*) with Thylate (thiram) in field tests conducted for 2 consecutive years in south Mississippi. The tests were carried out on an experimental planting of the Formosa variety arranged to facilitate disease investigations. Five replications were arranged in a randomized block design with 8 plants in each plot. These tests included only materials chosen after several years of study in the laboratory and greenhouse. The treatments (and concentrations per 100 gal. of spray mix) were: 1) a mixture of Dithane D-14 (1⅓ qt.), zinc sulfate (1 lb), lime (½ lb), and Triton B-1956 (1 fl. oz.); 2) Thylate (1 lb); 3) captan (2 lb) the first year and Phaltan, 75% (1¼ lb) the second year; 4) unsprayed check. Both Thylate and the Dithane D-14 gave excellent control of the disease and were significantly superior to the other materials. However, Thylate at 1 lb per 100 gal. of water provided longer protection, was less phytotoxic, and left less visible residue on the blossoms.

Fungicide-insecticide combinations for rye seed treatment.—*H. H. Luke, W. H. Chapman, and P. L. Pfahler.*—For the past 3 years rye grown in Florida has shown a reduction (40–50%) in germination; therefore, tests were initiated to identify the cause and to develop control measures. Initial tests indicated that *Helminthosporium sativum* was the pathogen involved and that ½ oz./bu of certain mercurial fungicides significantly increased grain yields. Recently it was observed that the rice weevil (*Sitophilus oryzae*) was involved. Tests were designed to develop fungicide-insecticide combinations that would control both organisms. Of 10 combinations tested monthly over a 4-month period, 4 increased germination and emergence 15–22%. The 4 most effective combinations were: 1) Ceresan M (½ oz./bu) and 5% DDT (1 oz./bu); 2) Delsan A-D (3½ oz./bu); 3) Ceresan 100 (½ oz./bu) and 50% malathion emulsion (2 oz./bu); 4) Orthocide Seed Protectant (1½ oz./bu). All treatments other than Ceresan 100 were applied in the slurry form. The Orthocide Seed Protectant caused slight root necrosis.

Studies on oat loose smut races of the Southeastern United States.—*H. H. Luke, S. J. Hadden, and D. D. Morey.*—Hosts that differentiate certain oat loose smut collections of the Southeastern United States were tested over a 4-year period under field conditions. Ten varieties that differentiated 8 different spore collections and 2 known races (A-14, A-15) were selected. Four collections exhibited consistent pathogenic reactions and were tentatively designated SA-1, SA-2, SA-3, and SA-4. SA-1 is similar to races A-15 and A-30. Limited field results indicated that SA-4 might be similar to race 10, but greenhouse results showed that the former would not parasitize Canadian; therefore, SA-4 is different from race 10. Additional greenhouse results indicated that SA-2 is similar to race A-20. SA-3 and SA-4 are apparently different from previously described races. SA-3 is differentiated from SA-4 by the susceptibility of Southland and Fulghum to SA-3, which are resistant to SA-4.

A top necrosis virus of beans from cantaloupe plants in the Rio Grande Valley.—*D. M. McLean.*—A necrotic, lethal virus disease caused an estimated 30–50% loss in October in about 800 acres of Blue Lake beans near Rio Grande City, Texas. Symptoms on beans were vein clearing with mild to severe coarse mottling on certain leaves, stunting, wilting, necrosis, and abscission of young leaves, terminal necrosis, necrotic stem lesions, yellowing of older leaves, marked stunting of growth, and ultimate death. The beans were planted following cantaloupes, and volunteer cantaloupe plants were growing among the beans. Mild "mosaic" symptoms were observed in the melons during the previous June, and "crown blight" symptoms were generally present. The virus from cantaloupes and the bean virus were indexed on a number of test plants. Both viruses caused ringspot symptoms in tobacco (Burley), mild chlorotic stippling and stunting in cucumber and cantaloupe, necrotic lesions on inoculated watermelons, systemic necrosis in beans, cowpeas, English peas, guar, and soybeans. Symptoms appeared 6–14 days after inoculation, but were masked in the cucurbits at high temperatures. The virus was transmissible mechanically and is thought to be a strain of the tobacco ringspot virus. A vector of the virus from cantaloupe to bean is not known.

Effects of soil fumigants on the occurrence of nematodes in field bins.—*Earl B. Minton, Eldon J. Cairns, and Albert L. Smith.*—Tests were conducted during 1957 and 1958 to determine the effectiveness of soil fumigants for eradication of nematodes in 20 field bins located on an Independence (Cahaba) loamy fine sand near Tallassee, Alabama. In November, 1957, 4 liquid nematocides injected 6 in. deep and MC-2 used alone and combinations of liquids with MC-2 did not eradicate nematodes to a depth of 31 in. Blanket retreatment of the bins in March, 1958, with a mixture of Nemagon, D-D, and Dowfume W-85 injected 6 and 18 in. deep followed by MC-2, 11 and 28 days later, reduced nematode population to a low level (15 nematodes in 628 soil samples). Two of the nematodes were found in the 0- to 10-in. sampling depth, and 13 in the 10- to 20-in. depth. The same bins were treated on June 10, 1958, with Telone, injected 12 in. deep, followed 10 days later by MC-2 to eradicate the survivors. A few *Trichodorus* contaminants were found in 14 bins 18 months after treatment. The data indicate that field bins can be freed of stylet nematodes by liquid fumigants injected at 6-, 12-, and 18-in. depths followed by intermittent applications of MC-2. Intensive sampling was necessary to detect the low numbers of survivors.

Population build-up and pathogenicity of reniform, root-knot, lance, and spiral nematodes on cotton, soybean, and tomato in field bins.—*Earl B. Minton, Albert L. Smith, and Eldon J. Cairns.*—Following eradication of existing nematodes in field bins, duplicate bins were reinfested in July, 1958, with single-species cultures of the following nematodes: *Meloidogyne incognita incognita, M. incognita acrita, Rotylenchulus reniformis, Rotylenchus brachyurus, Helicotylenchus nannus,* and *Hoplolaimus tylenchiformis.* Uninfested bins were used for controls. Plains cotton, Rutger's tomato, and Lee soybean were planted intermingled in rows throughout all bins in July, 1958. The following fall, Auburn woollypod vetch was planted, followed by 10 entries of cotton ranging from very susceptible to highly resistant to the cotton root-knot nematode. The average nematode counts per pint of soil 1 year after seeding the pure colonies were: *R. reniformis* 132,000; *R. brachyurus* 1,307; *M. incognita incognita* 456; *M. incognita acrita* 278; *H. tylenchiformis* 9; *and H. nannus* 7. The reniform nematode was pathogenic on all entries of cotton and caused stunting, delayed maturity, and reduced yield. Cultures of the two root-knot nematodes were found to be pathogenic to tomato and soybean, but were only weakly pathogenic to cotton. In 1959, *M. incognita acrita* stimulated growth, hastened maturity, and increased cotton yield.

Studies on seed-borne brown spot fungus in white lupine.—*Stanley A. Ostazeski.*—White lupine (*Lupinus albus*) heavily infected with the brown spot fungus (*Pleiochaeta setosa*) was observed at Quincy, Florida, in 1957. Diseased plants yielded a high percentage of discolored and scarred seed, which were divided into 4 groups by amount of discoloration: 1) sound, little, or no discoloration; 2) slight, less than ¼ of the seed coat discolored; 3) moderate, ¼ to ¾ of the seed coat discolored; 4) severe, more than ¾ of the seed coat discolored or scarred. *P. setosa* was isolated from 4, 14, 32, and 66% of the seeds sampled in Groups 1–4, respectively. Seed from groups 1–4 planted in methyl bromide fumigated soil respectively produced 2, 1, 5, and 20% diseased plants. Treatments in hot water at 50°C for 5–15 minutes preceded by a presoak treatment at 45° for 1 hour, eliminated the pathogen. Treatments in hot air at 75° for 1–8 hours shortly after harvest disinfected the seed. However, the hot-air treatment did not eliminate the fungus from seed stored at 7°C for over 1 year. Germination of healthy blue and yellow lupine seeds used as controls was not reduced by the hot-water and hot-air treatments.

Effect of exocortis disease on four citrus rootstocks.—*J. B. Sinclair and R. T. Brown.*—The effects of exocortis on the growth (trunk circumference), yields, and symptom expression of Washington navel on Cleopatra mandarin (*Citrus reticulata*), sweet orange (*Citrus sinensis*), trifoliate orange (*Poncirus trifoliata*), and Rangpur lime (*Citrus* sp.) for a 5-year period are summarized. That Cleopatra mandarin and sweet orange are adversely affected by the virus even though they exhibit no typical exocortis symptoms is indicated by the significantly (.01 level) slower growth in trunk circumferences of infected trees than that of healthy trees. Scions on rootstocks of trifoliate orange, Rangpur lime, and sweet orange showed a significantly (.01 level) reduction in yields of diseased trees below those of healthy trees, but there was no significant reduction in yields between healthy and diseased scions on Cleopatra mandarin rootstock. There was a tendency for an increase in the differences between the trunk circumferences of healthy and diseased trees on all 4 rootstocks, with the difference becoming wider each year.

Bacterial spot resistance of introduced peppers.—*Grover Sowell, Jr.*—Thirty commercial varieties and strains of pepper (*Capsicum frutescens*) previously reported to exhibit resistance to bacterial spot, caused by *Xanthomonas vesicatoria*, and about 600 pepper introductions were screened for resistance to this disease. The following tests were conducted: 1) a preliminary greenhouse screening test, 2) a replicated greenhouse test, and 3) a replicated field test. Isolate TS8 of *X. vesicatoria* was grown for 48 hours on fresh potato-dextrose agar (PDA) slants. Dehydrated PDA dextrose was not satisfactory. Petri-dish cultures were prepared from a suspension of the bacterium collected from the test-tube cultures and allowed to grow for 48 hours. Four- to eight-week-old seedlings were inoculated with a suspension containing about 1.5×10^6 bacterial cells per ml and incubated in a moist chamber for 48 hours. In the field test the varieties Santanka and Red Chili were more resistant to the disease than was California Wonder or Improved Truhart Pimiento. In all tests, the highest levels of resistance were shown by P.I. 163184, 163189, 163192, 164471, and 164677, all from India, and by the variety Anaheim.

Predacious activity and growth of nematophagous fungi on various organic substances.—*A. C. Tarjan.*—The nematophagous fungus *Dactylaria thaumasia* was cultured on 28 types of organic matter and was found to grow and sporulate best on either sugar cane filter press mud, corn bran, shredded peanut hulls, or oat hulls. This fungus proved most effective in reducing populations of the nematode *Panagrellus redivivus* in Erlenmeyer flasks when the source of organic matter was either sugar cane bagasse, soybean mill feed, ground oat hulls, corn bran, or stockyard manure. In a test in which *Dactylaria thaumasia*, *Arthrobotrys musiformis*, *A. oligospora*, *Dactylella ellipsospora*, and a *Dactylella* sp. were each added to flasks containing sugar cane filter press mud, ground oat hulls, unground oat hulls, and corn bran, and in which about 2500 *P. redivivus* were added to each flask after the fungus had sporulated, counts of surviving nematodes after 6 days showed *A. musiformis* to be the most voracious fungus and unground oat hulls to be the best organic substrate.

Some effects of African marigold on the citrus burrowing nematode, Radopholus similis. — *A. C. Tarjan.*—In 4 greenhouse tests, burrowing-nematode-infected grapefruit seedlings were interplanted with seedlings of African marigold, *Tagetes erecta*. In 3 of the 4 tests, there were substantially fewer nematodes in the soil 2½–6 months later than in soil containing infected seedlings but no marigolds. In only 2 of the tests, however, were numbers of nematodes in roots of citrus reduced by interplanting with marigolds. A fifth test used 3 boxes 4×1×2 ft. filled with autoclaved soil. In these, 12 uninfected grapefruit seedlings and 12 nematode-infected seedlings at opposite ends were separated by a zone 1×1½ ft., containing established marigold seedlings. After 8 months, nematodes from the infected citrus had passed through the barrier of marigold roots and penetrated roots of the previously healthy citrus. In a field test involving two 4-tree blocks of mature orange trees and 2 of grapefruit trees, each block 50 ×50 ft, 1 block of each citrus species was seeded with marigold. Twenty-five citrus root and soil samples were taken from each block 3 and 5 months after seeding, and 25 root samples were taken from each 7 months after seeding. There was no significant reduction of nematode population due to the presence of marigolds in any of the blocks.

Studies on the relation of fertilization to development of bacterial and Cercospora leaf spots in pepper.—*J. Taylor.*—In studies on the development of *Xanthomonas vesicatoria* and *Cercospora capsici* on pepper in the mountains of Georgia, the following fertilization rates were compared in 2 tests: an unfertilized check and N-P_2O_5-K_2O rates of 75-75-75, 150-150-150, 300-300-300, 600-600-600; and 1200-1200-1200 lb/acre. In one test, all plots were sprayed 3 times with ¾ lb Manzate (70%) plus 2 lb tribasic copper sulfate (53%) per 100 gal.; in the other test no sprays were applied. Bacterial spot development was similar in both tests during the first 6 weeks after transplanting. The average number of leaves lost per plant in the sprayed test was 19.6 for unfertilized plots and 12.2, 9.6, 6.6, 1.3, and 1.4, respectively, for plots with increasing rates of fertilization. There was no correlation between bacterial spot development and percentages of nitrogen, phosphoric acid, and potash in the pepper leaves. Cercospora leaf spot developed uniformly in the unsprayed test and almost completely defoliated plants in all plots by September. At the present stage of the investigation, the factors responsible for decrease in bacterial spot as fertilizer rates increase have not been determined.

Crease stem with bunchy top of tomatoes.—*P. A. Young.*—Summer tomatoes in 1959 showed numerous plants with the combined symptoms of crease stem and bunchy top. The symptoms were dwarfing, short internodes, some abnormal branching, rigidly upright stems, and compact bunchy appearance. One or more stems per plant showed flattened areas with longitudinal creases. Many kinds of tomatoes had shallow brown creases or sunken areas that extended into the stems. The creases in Rutgers variety were usually deeper and sometimes extended through the stems like slit windows. The pith was often browned under the creases. With few exceptions, plants with creases also showed bunchy-top symptoms. Blooming was delayed 1–2 weeks in Rutgers but not in STEP329 tomatoes. Bunchy-top symptoms became inconspicuous within a month, because of rapid growth of stems. Tomato varieties and selections ranged from immune to very susceptible to crease stem. The following percentages of plants showed crease

stem: Firesteel 2%, Greater Baltimore 43%, Hotset 4%, Rutgers 17%, Stokesdale 0%, STEP329 40%, and S1948 68%. Crease stem is a physiological abnormality associated with excellent growing conditions, especially abundant nitrogen and water.

Controlling southern blight of tomato with chemicals and crop rotation.—*P. A. Young.*—Southern blight (SB) caused by *Sclerotium rolfsii* killed all or nearly all of the tomato plants in preceding years in experimental fields of sandy land. Tomato plants with a ball of organic soil on the roots of each plant were used to test the chemically treated and untreated soil. Chemagro D-113, Vapam, and Mylone controlled SB well in the metal-bordered plots in 1959 until the end of the marketing season. Terraclor raked into soil at the rate of 516 lb per acre gave excellent control of SB but dwarfed the test plants. Injecting Terraclor at 53 lb per acre beside test plants controlled SB but temporarily decreased their yield. Different rates of Terraclor are to be tested. Calcium nitrate with 4 applications of 265 and 600 lb per acre did not control SB. *S. rolfsii* killed nearly all of the check plants in these experiments. In wet soil, *S. rolfsii* made thick collars of mycelium with sand around the bases of about 25% of the stems. Crop rotation during 6 and 8 years, mainly with Bermuda grass, crab grass and rye, controlled SB almost completely when ragweeds were hoed out. Farmers use fallow land to control SB by crop rotation. Southern blight was very destructive to tomatoes following corn or cotton rotation.

TURNIP MOSAIC VIRUS FROM TWO WEED HOSTS

W. N. Arnold and J. G. Bald

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SUMMARY

The virus in mosaic-infected *Brassica nigra* and *B. campestris* was studied because these weeds were once common around field trials of *Matthiola incana* progenies that were being tested for resistance against stock mosaic. Like stock mosaic, the Brassica isolates are forms of turnip virus 1. Only slight differences in symptoms were found between isolates. Inconsistencies in the reaction of Turkish tobacco were connected with differences in age or condition of inoculated leaves. A small percentage of turnip plants from commercial seed of the variety Purple Top Globe had a local necrotic reaction to the Brassica virus instead of systemic symptoms. A quantitative assay method for the Brassica virus was tested in dilution trials. It is based on systemic infection of mustard or turnip seedlings. It is reasonably accurate and economical of time and greenhouse space.

Walker et al (15) distinguished 2 groups within the aphid-transmitted viruses of crucifers: turnip virus 1 and cauliflower virus 1. Broadbent (3) in England distinguished members of the same 2 groups. Sylvester (12) further defined these groups and introduced a provisional third group, radish virus 1, that had properties between the other 2. Later work on a radish mosaic virus (4) has placed it with cauliflower virus 1, thus throwing doubt on the validity of Sylvester's intermediate group. Takahashi (13) described a virus disease of *Brassica nigra* (L.) Koch, the host range of which was extended by Sylvester, who placed it in the turnip virus 1 group. Tompkins' (14) mild and severe stock mosaic were also placed in this group.

Black mustard (*B. nigra*) and common yellow mustard (*B. campestris* L.) are prevalent weeds in southern California. Both species frequently exhibit coarse vein clearing, vein banding, and mottling. In spring of most years infected plants are common around the Ornamental Horticulture Area on the campus of the University of California, Los Angeles (UCLA), where Johnson and Barnhart (5) studied the inheritance of resistance to stock mosaic in *Matthiola incana* R. Br. They made field trials, dependent on natural infection presumably from nearby weeds carrying forms of turnip mosaic virus 1. Symptoms and host range of the *B. nigra* and *B. campestris* viruses and thermal inactivation temperatures have now shown that these 2 species carry a form of the turnip mosaic virus, differing only slightly on the one hand from Takahashi's *B. nigra* isolate, and on the other from Tompkins' mild stock mosaic. Infection-dilution trials have shown the degree of accuracy possible in estimating relative virus concentration from numbers of systemically infected seedling test plants.

Materials and methods. All the virus isolates used in this work were originally from diseased plants on the UCLA campus. Young infected plants were dug from the hillside, potted, and held in a lath house. The *B. campestris* specimens flowered and identification was confirmed. Three specimens of *B. nigra* were used in the preliminary inoculation trial; afterward only one of the 3 was maintained, and it died before flowering. An identification was made on the basis of plant habit and the form and distribution of leaf hairs. The virus isolates from the field were also transferred to and maintained in turnip and mustard.

Test plants for host range determinations were grown in U.C.-type soil mix contained in 4- or 5-in. unglazed porcelain pots. When large numbers of seedlings were required for physical studies they were grown directly from seed in small peat "Jiffy" pots.

Inoculum was prepared by grinding leaf tissue in a mortar with phosphate-citrate buffer of pH 7.0. The crude juice, or the supernatant from slow-speed centrifugation, was applied to carborundum-dusted leaves with a ground-glass spatula (10). The virus inactivation temperature was determined in the usual way. Inoculum in thin-walled test tubes was heated to several temperatures by immersion in constant-temperature water baths. Twelve seedling test plants were inoculated from each virus sample.

Results. — *Host range.* — Ten selected species of *Cruciferae*, 4 *Solanaceae*, and 2 *Chenapodiaceae* were inoculated with a number of the UCLA Brassica virus isolates. Host ranges agreed closely, but not abso-

lutely, on the one hand with mild stock mosaic virus (14) and on the other hand with Takahashi's *B. nigra* virus. Unlike the former, the UCLA isolates failed to infect radish and *N. glutinosa*. Whereas Takahashi's *B. nigra* virus (12, 13) gave no symptoms on tobacco, some UCLA isolates gave a few necrotic local lesions, and others none, and the response of several isolates varied on different occasions. A reasonable explanation for these inconsistencies appeared in the following trial:

Nine of the UCLA *B. nigra* and *B. campestris* isolates were inoculated to small actively growing Turkish tobacco plants. On some, the third to the fifth leaves above the cotyledons were inoculated, and on others the fourth to the sixth leaves. Expanding necrotic lesions appeared on at least 1 bottom inoculated leaf in all 9 series. On the second inoculated leaf, lesions were smaller, and on the third they were few and small or absent. Numbers of lesions on the oldest or the middle leaves varied from 0, 1, or 2 to 80–100, indicating radical differences of virus concentration in the turnip plants that supplied the inoculum. Old fully developed turnip plants contained the lowest concentrations of virus. The reaction of tobacco to some forms of turnip virus 1 thus fails to give a reliable distinction between strains. Since it is the only host-range difference detected between Takahashi's *B. nigra* virus (13) and the UCLA isolates, they may be regarded as essentially similar in host-range reactions.

On *Matthiola incana* the UCLA isolates caused typical symptoms. A whiteflower variety was inoculated with several Brassica isolates, but remained vegetative for 7 months before flowering because the test plants did not receive sufficient treatment at cool temperatures to cause flower-bud initiation. The upper leaves developed mild mottling, with or without some twisting or distortion. A colored variety, Dwarf 10-week Crimson (Denholm Seed Co.,), was forced into flowering by cool temperatures and inoculated with 11 of the Brassica isolates when it was in the early bud stage. All isolates produced severe flower breaking, some flowers being almost white. On later-emerging flowers the symptoms were less severe. The only foliar symptoms were a faint chlorosis and very slight stunting.

Variations in host range between isolates of turnip virus 1 inoculated side by side are common (6). There is no evidence that the minor differences in host range between Tompkins' mild stock mosaic (14) and the UCLA Brassica viruses are related to the host species from which they were originally isolated. The latter appear equally capable of causing mild mosaic disease in stocks, particularly the flower-breaking symptom.

Virus properties.—The heat inactivation temperature of representative UCLA Brassica isolates lay between 53 and 63°C, the limits reported for various strains of turnip virus 1 (6, 12). Members of the cauliflower virus 1 group are inactivated at temperatures above 70°C.

The survival time *in vitro* of the UCLA Brassica virus was not determined under conditions similar to those for determinations by other workers. During inoculations of seedlings with serial dilutions of virus, there was no evidence of reduction in activity of the inoculum after 1–2 hours. Extract from an infected turnip leaf, which had been stored in a deep freeze under nitrogen for 3 weeks, gave 100% infection on 10 mustard seedlings tested.

Necrotic reaction of turnip plants.—The initial symptoms produced by the UCLA isolates on fully expanded leaves of the turnip variety "Purple Top Globe" was a coarse vein clearing typical of the turnip virus 1 group. On occasion some seedling plants exhibited systemic necrosis of stems and leaves. A trial was designed to find whether this aberrant symptom was due to a mixture of strains in the inoculum or to a difference in reaction between plants within the turnip variety.

In one of the infection-dilution trials yet to be described, the most highly diluted extract produced infection in 4 of 20 inoculated turnip seedlings. One of the 4 plants exhibiting only necrotic lesions provided the so-called "necrotic source," and 1 of the other 3 plants, with mottle only, provided the so-called "mottle source." The test plants fell into 2 age groups (14 and 28 days after sowing). The results are presented in Table 1. Virus from the mottled and necrotic source plants, reinoculated to turnip, cause about the same proportion of necrotic reactions. The different symptoms are due to variation between plants within a variety rather than in the virus. The older plants are more likely to have a necrotic response to infection than the very young seedlings. For the totals, 35-3 (young plants) and 13-8 (older plants), $\chi^2 = 5.93$. A proportion of necrotic reactions in the variety Purple Top Globe continued to appear in other experiments.

Quantitative studies, dilution trials.—Since McKinney's (8) original work on quantitative methods with plant viruses, little use has been made of whole plants of a susceptible species for assaying virus concentration. Often, lacking good local-lesion hosts, no attempt is made at quantitative studies with a virus. Unpublished work (J.G.B.) has shown that for certain purposes systemic hosts are useful and desirable in quantitative studies, even with some strains of tobacco mosaic virus. In the present work no good local-lesion host for the UCLA Brassica virus was found among the host species inoculated, so systemic hosts were used for quantitative studies. Since similar situations are not uncommon in virus work, the results of dilution trials with these isolates of turnip virus 1 are given below.

Table 1.—Symptom expression on *Brassica rapa* var. Purple-top Globe inoculated from turnip seedlings with mottle and necrotic reactions to an isolate of the Brassica virus.

	Mottle	Necrosis
"Necrotic source" (14-day plants)	17	1
"Necrotic source" (28-day plants)	7	4
Total	24	5
"Mottle source" (14-day plants)	18	2
"Mottle source" (28-day plants)	6	4
Total	24	6

Table 2.—Number infected of 20 seedling plants inoculated at various dilutions of infected plant tissue. M. indicates mustard source or test plants; T indicates turnip.

Dilution series	1	2	3	4	5	6	7	8	9	10	11
Source host plant	M	M	T	M	T	T	T	T	T	T	T
Test plant	M	M	T	T	T	T	T	T	T	T	T
Dilution, 1 part in:											
3.3			18								
4							13	12	13		
5	20										
10		17	15		15	19					
12.5										11	6
33			15							8	3
40							6	3	5		
55	15										
71										6	2
100		20	5	15	8	16					
169										5	1
200				14	11	10					
330			2								
400							0	1	2	1	0
550	8										
1000		8		2	2	2					
2000				1							
3300			1								
4000							0	0	1		
5500	4										
10000		2			1	0					
20000		2									
55000	0										

Seedlings for quantitative tests were grown by a modification of methods devised by Bald and Chandler [see Matkin and Chandler (7)] to obtain uniform tobacco plants. Several seeds of mustard or turnip were sown in each of 100 or more 2½-in. peat "Jiffy" pots. All but 1 seedling per pot were rogued. As far as possible those emerging on one day were retained; otherwise the seedlings were matched for age and uniformity, and treatments were randomized within groups of equivalent plants. Inoculations were made on the 2 cotyledons of each seedling the seventh to tenth day after seed was sown, i.e., before or soon after the first true leaf emerged. In all dilution trials, 20 seedlings were used for each sample; in the later trials these were divided into 4 replicate lots of 5 before inoculation.

Seven experiments including 11 dilution series were made (Table 2) with various of the Brassica virus isolates. The results of 4 dilution series are plotted as log per cent infected plants against log dilution of infected leaf tissue in the inoculum (green weight); for 2 series (Table 2-1,2 and Fig. 1) both source plants and test seedlings were mustard, and for the other 2 (Table 2-3,5 and Fig. 1) they were turnip. The 4 series were obtained independently. A freehand curve of the same form is drawn through both sets of points at different positions along the x-axis; the points for mustard fall about the curve at one position, and the points for turnip fall about the curve at the other position. The concentration of virus in the source mustard plants appeared similar on 2 occasions, and so did the concentration in the source turnip plants. The main factor in the shift of the points along the x-axis from the mustard series to the turnip series was apparently the lower sensitivity of turnip seedlings to infection by mechanical inoculation.

The form of the dilution curves was similar in 7 of the 11 series (Table 2-1, 2, 3, 5, 8, 9, 10). The curves sloped at relatively low angles over wide ranges of virus concentration. If infection had occurred from discrete virus particles randomly distributed on inoculated leaves, a steeper slope (Fig. 1, top curve) might have been expected (1). Such deviations from the expected form of the dilution curve of tobacco mosaic virus have been attributed to aggregation of virus particles at higher concentrations, and dispersion of the aggregates on dilution (2). Preliminary electron micrographs of the Brassica virus demonstrated *in vitro* virus aggregation, though preparations were made at a dilution that gave very few particles on each screen. Takahashi's illustration (13) also suggests aggregation.

Four of the dilution series approached more closely to the theoretical form of curve (Table 2-4, 6, 7, 11). Numbers 6, 7, 11 were inoculated in parallel with isolates that gave curves of lower slope, like those illustrated (Fig. 1). Comparing series 7, 11, and 8, 9, 10, infections as square root of n + 1 for dilutions 1:4 to 1:400 were fitted with straight-line regression curves. A difference in slope significant at the 1:20 level of probability was demonstrated. Further work with single-lesion isolates is needed to show whether differences in the slopes of dilution series are linked with the tendency of the virus particles to aggregate *in vitro*.

Quantitative results.—Statistical analysis showed, for comparisons between groups of 20 seedlings, that the least significant difference was about 5 infected seedlings, i.e., about 25% of the plants inoculated. One hundred seedlings per treatment would have given the same degree of accuracy as a TMV local-lesion trial. Considering the time needed to grow the plants and complete a trial (about 4 weeks) and the bench space

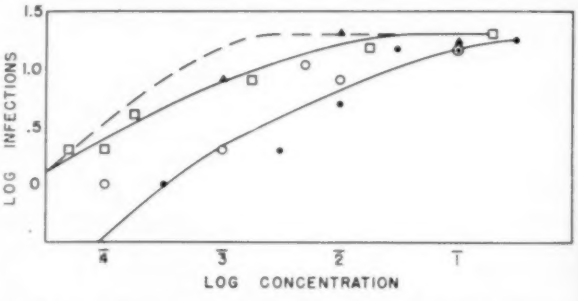

Fig. 1.—Data from 4 dilution trials with UCLA Brassica virus isolates. Log numbers of infected plants of 20 inoculated (y-axis) plotted against log concentration of inoculum (x-axis). The points grouped about the upper solid line represent inoculations from mustard to mustard (2 trials), those grouped about the lower solid line, from turnip to turnip (2 trials). The broken line represents a theoretical dilution series of the form expected from fully disperse virus distributed at random over uniform inoculated leaves.

(slightly more than 4 sq ft per 100 seedlings), the accuracy of this method is commensurate with the means used to attain it.

Theoretically the whole-plant method has the same basis as the local-lesion method (1), but it has the advantage that the upper limit of infection, 100%, may be given automatically by the number of inoculated plants. When, as here, there is evidence of departure from the direct relationship between infections and numbers of virus particles, means may be found (9, 11) to use the slope of the dilution curve in estimating relative virus concentration.

Discussion.—These experiments have given answers to 2 questions raised by Johnson and Barnhart's work (5) on resistance in stock, *M. incana*, to the mosaic disease that causes flower breaking in commercial plantings. The virus in the weeds, *B. nigra* and *B. campestris*, from which infection of field plantings presumably arose, is capable of producing typical flower breaking in stock, and isolates from the 2 weed hosts have the characteristics of the turnip virus 1 group as defined by Sylvester (12) and others. The experiments also demonstrate how easily the physiological state and developmental stage of test plants and the concentration of virus inoculum may introduce minor discrepancies in virus host ranges.

The existence of turnip plants with a necrotic reaction to infection, grown from seed of a susceptible variety, raises the question whether the seed used was pure, and whether, if pure, it carried genes giving it a necrotic reaction to infection by the turnip mosaic group of viruses. Whichever explanation applies, there seems to be a possibility of obtaining turnips with field resistance to mosaic disease, provided the virus strains attacking turnip produce the same effect, and the necrotic reaction is a heritable character. So far as the authors are aware, such a reaction of turnip to turnip mosaic virus has not previously been reported.

The quick germination of mustard seeds and the ease with which they may be infected with the Brassica virus in the cotyledon stage make mustard an excellent systemic host for use in quantitative work. Turnip is usable, but not so sensitive. The efficiency of the whole-plant method depends on adequate techniques of growing, matching, and inoculating the seedling plants.—Department of Plant Pathology, University of California, Los Angeles.

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FUNGICIDE VAPOR ACTION, INEFFECTIVE IN WHEAT SMUT CONTROL BY SEED TREATMENT

Laurence H. Purdy and C. S. Holton

Pathologists, Crops Research Division, ARS, U.S.D.A., Pullman, Washington.

Cooperative investigations of the Crops Research Division, ARS, U.S.D.A., and the Agricultural Experiment Stations of Idaho, Oregon, and Washington.

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SUMMARY

Five seed-treatment fungicides, including 4 mercury products and 1 non-mercury preparation, were tested to determine their relative effectiveness in controlling wheat smut by vapor action. Various degrees of slight control by some of these materials were indicated, but it was evident that fungicidal vapor action is not a significant factor in the control of wheat smut by seed treatment in the Pacific Northwest.

The acceptance of organic mercury preparations as seed-treatment fungicides for controlling certain diseases of seed-borne origin focused attention on the importance of volatility in the total fungicidal action of these materials. Although direct contact with the pathogen is usually a primary requirement for the disease-control action of organic mercurials, their vapors also are purportedly fungicidal to many plant pathogens, including *Tilletia* spp. on wheat. Hence, one re-

Table 1.—Average smut percentages obtained in tests of the effectiveness of fungicide vapor action in controlling bunt of winter and spring wheats by seed treatment.

Treated: untreated seed	Smut % at single and double rates[a] of fungicides indicated											
	Panogen 15		Ceresan 75		Ceresan M		Ortho L M		Anticarie		Check	
	0.75	1.50	0.75	1.50	0.5	1.00	0.75	1.50	1.00	2.00		
95:5	31	38	29	30	28	36	28	17	16	0	50	92
90:10	27	52	36	47	33	51	47	44	11	4	66	97
80:20	38	82	41	77	32	80	48	81	11	35	62	80
70:30	43	85	43	76	36	64	55	80	19	35	63	86
60:40	53	77	46	78	44	80	55	75	19	48	62	95
50:50	53	82	49	90	55	82	52	83	28	65	65	93
Average	40	69	40	66	38	65	47	63	17	31	61	90

[a] 0.75 and 1.50 are respectively single and double rates.

quirement of the standard seed-treatment process for wheat smut control is to hold treated seed under a covering or in large piles at least 24 hours before planting. Presumably, in this period the fumigant action compensates for lack of direct contact between fungicide and smut spores on seeds that carry no fungicides. Indeed, the basic operating principle of the slurry-type seed-treating machine was apparently predicated on the premise of this fumigant action, since the limited mixing it provides can scarcely distribute to each kernel its share of the small amount of mercury fungicide recommended for a bushel of seed. Consequently, advent of the relatively non-volatile chemical hexachlorobenzene (HCB) for wheat smut control in the Pacific Northwest was viewed with some skepticism, especially by proponents of mercury fungicides. For this reason the fungicide vapor action in seed treatment for wheat smut control in the Pacific Northwest has been under scrutiny for several years.

In 1956 (6, 8) it was reported that Panogen 15 [cyano(methylmercuri)guanidine] controlled seed-borne common bunt (*Tilletia caries* (DC.) Tul. and *T. foetida* (Wallr.) Liro) by vapor action, whereas Ceresan M [*N*-(ethylmercuri)-*p*-toluenesulfonanilide] and Anticarie (hexachlorobenzene) failed to provide control by such action. These results were from experiments in which the seed-borne smut spores were exposed to 2.5–25 times the vapor concentration potential of commercial treating operations. Furthermore, the method of exposing the inoculated seed to the vapor action of the fungicides failed to simulate conditions encountered in commercial seed-treatment operations. Additional experiments were undertaken in which the efficacy of the vapor action of several different fungicides was compared under conditions simulating commercial seed-treating conditions. The results are reported herein.

Materials and methods.—The fungicides tested were 3 liquid mercury preparations—Panogen 15, Ceresan 75 [2.8% ethylmercury 2,3-dihydroxypropyl mercaptide and 0.6% ethylmercury acetate], and Ortho L M Seed Protectant [2.4% methylmercury 8-hydroxyquinolate]—and 2 wettable powders—Ceresan M and Anticarie. The liquid fungicides were used in concentrated form (without dilution in water), and the wettable powders were applied as slurries. The tests were conducted on Orin winter wheat and Red Bobs spring wheat, which were respectively inoculated with spores of races T-16 and T-5 (9).

To test the control action of their vapors, the fungicides were carried on uninoculated seed mixed with inoculated untreated seed. In the winter-wheat tests, the fungicide carrier was spring wheat, and in the spring-wheat tests, it was winter wheat. Spring wheat does not survive the severe winter conditions, and winter wheat does not head from spring plantings. Consequently, only the inoculated seed produced wheat plants that headed out under the 2 different planting conditions, and the only possible control action was from vapor action, plus a minimum of direct contact between treated and untreated seed.

To test for possible relationships between degree of vapor action and fungicide volume, treated and untreated seed were mixed in 6 different proportions (Table 1). The total volume of seed for each mixture was 20 ml, and the different proportions of treated and untreated seed were based on this volume. For example, the mixture of 90:10 contained 18 ml of treated seed and 2 ml of untreated seed. A check series with the same mixture proportions was made up with uninoculated untreated seed and inoculated seed.

To provide for maximum fumigant action of the fungicide vapor, each 20-ml sample of seed was placed in a 20-ml screw-top vial with the top fitted tightly to prevent escape of the vapors. All vials were held 72 hours at 15°C, after which the seed from each vial was divided into 3 equal lots and planted in field or greenhouse.

Eight different tests were completed in 1957–59 with fungicides applied at the commercial rates recommended for bunt control (single rate). Two were winter-wheat tests, 2 were spring-wheat tests in the field, and 4 were spring-wheat tests in the greenhouse. Each treatment was replicated 3 times.

The results were measured in percentage of infected heads in each test row.

Results.—Since the results of each experiment generally confirmed those of previous ones, all results are presented as averages in Table 1. Smut control was slight or absent from the highly volatile mercury compounds, and significantly effective from the relatively

non-volatile HCB preparation (Anticarie). In the experiments with single rates of application, average infection was 38–47% for the 4 mercury compounds, compared with 61% in the check. The hexachlorobenzene preparation reduced infection to 17%. In tests with double rates the smut averages were 63–69% in mercury treatments, 31% in HCB, and 90% in the check. That these are higher than the percentages in the single-rate tests is attributable to more favorable conditions for infection rather than to rate differences.

The double rates were tested to compensate for the reduced vapor-action potential inevitable in a unit lot of mixed treated and untreated seed, as compared with a comparable unit of seed composed entirely of treated seed. Thus, in a 1:1 unit of seed the vapor action potential is only 50% at the single rate and 100% at the double rate. Even the double rate gave little or no smut control by any of the mercury treatments (Table 1). Anticarie, in contrast, reduced infection markedly in all tests, being particularly effective at the double rate in the 95:5 and 90:10 seed lots (Table 1).

It is not conclusive, however, that fungicide vapor action was entirely responsible for even the slightly reduced infection percentages in these experiments, since some direct contact between fungicide and inoculum is inevitable in seed lots of treated and untreated seed. In other words, like those of earlier experiments (6, 8), these results do not support the prevailing conviction that vapor action of seed-treatment mercury fungicides is important in wheat smut control. This conclusion does not imply, however, that the vapor action of mercury fungicides is not effective in controlling other diseases of seed-borne origin. Certain mercury preparations commonly used as seed-treatment fungicides are highly volatile, and there is evidence that their products exert toxic effects on certain seed-borne pathogens (1, 2, 3, 4, 5). None of this evidence applies to wheat smut control, for which maximum direct contact is the all-important consideration. Maximum direct contact is obtained when the fungicide is uniformly distributed throughout the seed mass and envelopes the individual kernels (7).—Regional Smut Research Laboratory, Washington Agricultural Experiment Station, Pullman, Washington.

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EFFECT OF TEMPERATURE, pH, AND VARIOUS NUTRIENTS ON GROWTH OF CLADOSPORIUM CUCUMERINUM

D. L. Strider and N. N. Winstead

Respectively, Research Assistant Professor of Plant Pathology, Mountain Horticultural Crops Research Station, Fletcher, North Carolina, and Associate Professor of Plant Pathology, North Carolina State College, Raleigh.

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SUMMARY

After 8 days in a liquid medium, *Cladosporium cucumerinum* grew over a temperature range of 2–28°C. Temperatures of 30° and above were lethal. Growth of the organism was observed in the same medium buffered with citric acid over a pH range from about 2.5 to 9.0 with an optimum between 3.5 and 4.0. When citric acid was replaced with $10^{-2}M$ acetic acid, the pathogen failed to grow at or below its pH optimum as determined on the citric-acid-buffered medium. This toxic effect was highly pH dependent.

C. cucumerinum utilized many different carbon and nitrogen sources. The pathogen utilized each of 22 amino acids tested as nitrogen sources except cysteine. Cysteine was toxic at a concentration between 10^{-3} and $10^{-4}M$ in the presence of KNO_3. In the absence of KNO_3, no growth occurred on cysteine at any concentration tested, including $10^{-5}M$. Better growth occurred with asparagine than with a combination of asparagine and KNO_3 as nitrogen sources. Best growth occurred on aspartic acid with or without KNO_3.

Introduction.—*Cladosporium cucumerinum* Ell. & Arth. is the incitant of a disease of considerable economic importance on many of the Cucurbitaceae. In the mountains of North Carolina, the severity of this disease largely determines the success or failure of late-planted summer squash, *Cucurbita pepo* var. *melopepo* (L.) Alef. (1). Scab is a destructive disease of cucumber, *Cucumis sativus* L., wherever environment favors the pathogen, and is particularly important in northwestern Europe, northern United States, south-

ern Canada, and the mountains of North Carolina. This organism incites the most important transit and storage disease of muskmelon, *Cucumis melo* var. *reticulatus* (L.) Naud. (7), and is one of the limiting factors in the production of this crop in western North Carolina (8).

Since foliage fungicides have not been very effective for scab control on cucumber, prime dependence has been on the development of resistant varieties. Many scab-resistant cucumber varieties have been developed through breeding and selection, but the chemical nature of the resistance is unknown. The nutrition-inhibition hypothesis of Garber (2) holds that a plant is susceptible to a pathogen when the suscept presents a suitable nutritional environment and fails to present an inhibitory environment for growth of the pathogen. The present study attempts to determine some of the nutritional and inhibitory environments of *C. cucumerinum in vitro*. Specifically, the objectives were to determine: 1) the effects of temperature and pH on growth of the fungus *in vitro*; 2) whether the organism could utilize certain carbohydrate sources for growth; and 3) the effect of various amino acids and inorganic compounds on growth and sporulation.

Muyzenburg (5) found that growth of the pathogen on agar was restricted to a range of 0–32°C, with optimum at 21°. Heringa-Westerhof (3) reported good growth of the fungus at pH of 5–8. No studies on the nutrition of *C. cucumerinum* were found in the literature.

Materials and methods.—The isolate of *C. cucumerinum* used was originally from a diseased squash fruit from the mountain area of North Carolina and maintained by the Plant Pathology Department of North Carolina State College. The cultures used were subcultures from a single-celled conidium isolated from this culture. The isolate remained virulent throughout the study as evidenced by inoculation tests on Stono cucumber seedlings at various intervals.

The effect of temperature on growth of *C. cucumerinum* was studied on solid media and in liquid culture. The solid substrate was PDA buffered with K_2HPO_4 (4.0 g), citric acid (15.0 g), and glycine (6.0 g) per L of medium, adjusted to pH 4.0 with *N* HCl. About 30 ml of the medium was poured into each of 6 Petri plates for each temperature tested: –8, 2, 4, 8, 12, 16, 20, 24, 28, 30, 32, 34, and 36°C. Spores were transferred from a 5-day-old PDA culture with the point of a transfer needle to the center of the medium in Petri plates. Colony diameter was used as the measurement of growth and observations were made at 4, 8, and 12 days. The plates at the extreme temperature values were transferred after the 12-day interval to 24°C to determine viability.

The liquid medium was: bacto-casamino acids (1.0 g), K_2HPO_4 (4.0 g), $MgSO_4$ (0.1 g), $FeCl_3$ (0.02 g), sucrose (10.0 g), citric acid (15.0 g), glycine (6.0 g), and distilled water (1.0 L), adjusted to pH 4.0 with *N* HCl. Growth was determined by measuring the dry weight of mycelium produced in still liquid cultures in 40 ml of medium contained in 125-ml Erlenmeyer flasks. Inoculum was obtained from 5-day-old PDA cultures. The spore concentration was adjusted so that 1 ml of spore suspension added to 40 ml of medium gave a final concentration of about 10^3 spores per ml of medium. The cultures thus prepared were placed at 24°C in complete darkness. Three flasks were prepared for each temperature and each observation interval (identical to those of the temperature test on solid medium). After a growth interval the mycelium was dried on filter paper for 18 hours at 95–100°C.

The materials and methods adopted to study the effect of pH on growth of the pathogen were similar to those used in the temperature study except as noted below. In certain of the pH studies 15.0 ml of acetic acid was substituted for citric acid. Each of the constituents of the liquid medium was dissolved separately, then thoroughly mixed. The medium was adjusted to the 16 desired pH values with *N* HCl or NaOH. Thirteen 125-ml flasks were then prepared for each pH value tested. These values were generally at 0.5 pH unit intervals from 2.0 to 11.0 and were attained before autoclaving. One flask at each pH value was used to determine the initial pH after autoclaving. Four flasks were used for each pH value and at each of the 3 observation dates. Three of the 4 flasks in each case were seeded with a spore suspension as described

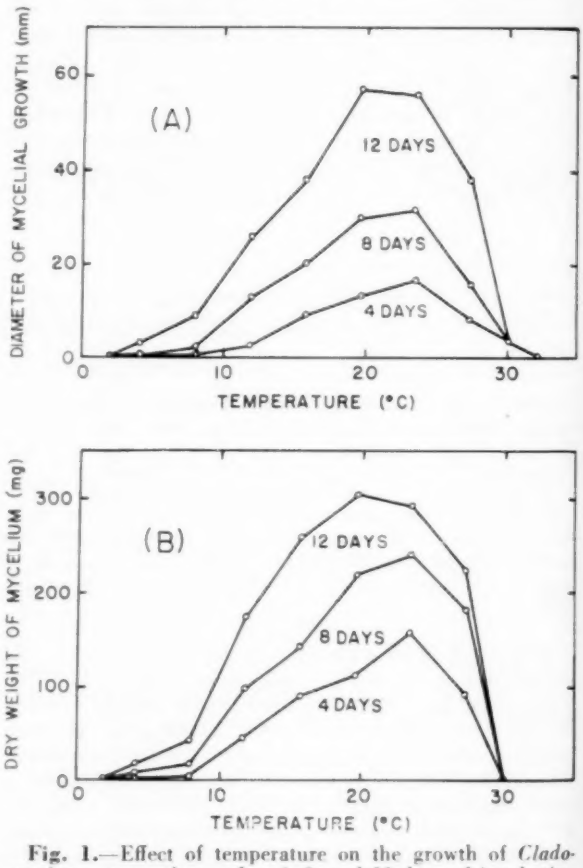

Fig. 1.—Effect of temperature on the growth of *Cladosporium cucumerinum* after 4, 8, and 12 days of incubation on: **A)** a solid medium; **B)** a liquid medium.

previously, and the fourth was used as a check on the pH stability in the absence of the organism.

Also conducted was a pH study on PDA, buffered as in the temperature studies. The technique used for obtaining the various pH values was similar to that described previously.

The basal medium used for the carbohydrate and nitrogen source studies was: K_2HPO_4 (1.0 g), KCl (0.5 g), $MgSO_4$ (0.5 g), $FeSO_4$ (0.01 g), and distilled water (1.0 L). The carbohydrates tested were: dextrose, maltose, lactose, galactose, sucrose, levulose, and soluble starch. The nitrogen compounds tested were: $NaNO_3$, NH_4NO_3, $(NH_4)_2SO_4$, KNO_3, bacto-casamino acids, urea, asparagine, and casein. The nitrogen compounds were used at the rate of 1.0 g per L of medium and the carbohydrates at 30.0 g per L of medium. Each carbohydrate was tested in combination with each nitrogen compound, giving 56 treatments. Each treatment was replicated 3 times. The pH of each treatment was adjusted to 7.0 before autoclaving. Forty ml of a medium was poured into 125-ml flasks, seeded with a spore suspension, and incubated 8 days at 24°C.

The basal medium used for studies on the effect of various amino acids on growth and sporulation of the pathogen was a Richard's solution minus nitrogen. The amino acids (Table 2) were tested individually and in combination with KNO_3. The 2 concentrations of amino acids were 0.3 and 0.1 g of nitrogen per L of media. Where the amino acids were tested in the presence of KNO_3, the KNO_3 concentration was equal to that of the amino acids in terms of g of nitrogen per L of media. Bacto-casamino acids at 0.3 and 0.1 g of nitrogen per L of medium were used as a control. A check treatment containing no nitrogen was also used. Five ml of medium was transferred to each of four 30-ml test tubes for each treatment and autoclaved. The media were seeded with 1 drop of a spore suspension and incubated 6 days at 24°C. Microscopic observations were made to determine whether the growth produced by one treatment was less than, equal to, or greater than that produced on the basal medium plus KNO_3. Microscopic observations to determine the relative amounts of sporulation were also compared with the basal medium plus KNO_3.

Results.—*Effect of temperature on growth.*—After 12 days, growth was measurable on the solid PDA medium in the temperature range of 2–30°C. The cultures at 32° failed to grow and did not begin growth when transferred to 24°C. Cultures at 2 and –8°C produced abundant growth after transfer to 24°C. The cardinal temperature values for growth of the fungus in 12 days were 2, 20, and 30°C (Fig. 1-A). The fungus grew more rapidly at 24° than at 20° for the first 8 days, but at 12 days the largest colonies were at 20°C. In all cases differences in colony diameter between 20° and 24° were slight.

On the liquid medium, growth occurred at all temperature intervals from 2 to 28°C but no macroscopic mycelium occurred at 30°. The cultures incubated 12 days at 30° or above failed to begin growth when transferred to 24°; the cultures at –8°C, however, grew when transferred to 24°. Subsequent tests with for the same medium showed that the organism was no longer viable after 24 hours at 32°C or 72 hours at 30°C. The cardinal temperature values for growth of the organism in these tests were 2, 20, and 28°C (Fig. 1-B). Again, colonies were larger at 24° than at 20°C at 4 and 8 days. After 12 days at 24°, the cultures were obviously beginning to deteriorate.

Effect of pH on growth.—In studies of the effect of pH on growth, the basal medium was buffered with 1.5% acetic acid. Growth of the fungus was observed on media with pH values after autoclaving of 6.3–9.0. After 8 days no macroscopic mycelium was observed on media adjusted to pH 6.0 or below (Fig. 2). The 4- and 12-day observations yielded data similar to that obtained at 8 days. Transfers to PDA were made from the 4-day-old cultures at pH 6.0 and below and placed at 24°C. No colonies were observed on any of these plates after 8 additional days of incubation. Since the organism did not grow at pH 6.0 or below when acetic acid buffer was used, these studies were repeated with a basal medium buffered with 1.5% citric acid. Table 1 shows the original pH values before and after autoclaving, along with the values recorded at the 8-day observation. The pH values of the culture medium were essentially the same at 4 days as after autoclaving. Growth of the fungus was greatest in media with an initial pH after autoclaving of 3.5. This was true for both 4- and 8-day observations. Fig. 2 shows the dry weight of mycelium produced by the organism over the pH range tested. Colonies of the fungus grew on PDA transfers from cultures at each pH value tested.

On the PDA medium, colonies were largest at an initial pH after autoclaving of 2.8. The initial pH values and the changes occasioned by autoclaving and 8 days' incubation were very similar to those presented in Table 1. The growth curve obtained by plotting colony diameter against the pH values tested was very similar to that for the citric-acid-containing medium (Fig. 2).

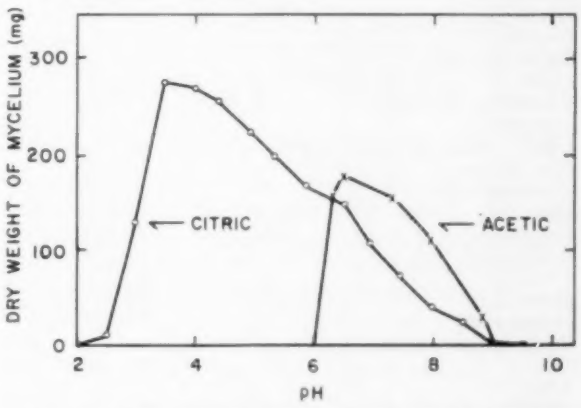

Fig. 2.—Effect of pH on the growth of *Cladosporium cucumerinum* after 8 days in a basal medium containing 1.5% citric acid or 1.5% acetic acid. The growth increments are plotted at initial pH values after autoclaving.

Growth on various carbohydrate and nitrogen sources.—The organism grew on each of the 56 combinations of carbohydrate and nitrogen tested. Growth was best in media containing casamino acids as a nitrogen source. Growth was very poor in all media containing either starch or casein.

The fungus was able to use, as the sole source of nitrogen, each amino acid tested except cysteine. Cysteine was toxic to the organism at the lower concentration tested (slightly less than $10^{-2}M$). Spores of the fungus failed to germinate on media containing cysteine but germinated on the medium devoid of nitrogen. Growth was also inhibited by the other 2 sulfur-containing amino acids cystine and methionine, and by leucine. Growth was best on the aspartic acid treatments, with and without nitrate. Growth was better when nitrogen was supplied as asparagine alone than as nitrate and asparagine. Sporulation was greatest on the asparagine treatments without nitrate. With the exception of glycine, growth at the lower level of amino acid nitrogen was equal to or greater than that at the higher level of amino acid nitrogen tested. Table 2 lists the various treatments, their relative effects on growth, and the relative degrees of sporulation at both levels of amino acid nitrogen used.

Since no colonies were observed on the cysteine treatments in the preceding test, the toxicity of several concentrations of cysteine was investigated with the same basal medium. Six replications of each of the following treatments were prepared: 10^{-3}, 10^{-4}, and $10^{-5}M$ cysteine, with and without 0.3 g per L of inorganic nitrogen in the form of KNO_3. The check contained no nitrogen. All media were adjusted to pH 4.0 and autoclaved for 6 minutes. Observations

Table 1.—pH values of the basal medium containing 1.5% citric acid, and of the medium and cultures seeded with *Cladosporium cucumerinum* after 8 days of incubation.

Before autoclaving	After autoclaving	Medium at 8 days	Filtrate at 8 days
2.0	Unchanged	Unchanged	Unchanged
2.5	Unchanged	Unchanged	Unchanged
3.0	Unchanged	Unchanged	Unchanged
3.5	Unchanged	Unchanged	6.9
4.0	Unchanged	Unchanged	7.5
4.5	Unchanged	Unchanged	6.4
5.0	Unchanged	Unchanged	7.6
5.5	Unchanged	Unchanged	7.8
6.0	Unchanged	Unchanged	8.1
6.5	Unchanged	Unchanged	7.9
7.0	7.1	7.1	7.4
7.5	7.8	7.7	7.4
8.0	8.3	8.1	7.7
8.5	8.7	8.4	7.7
9.0	9.0	8.7	8.7
9.5	9.5	9.0	9.0
10.0	10.0	9.2	9.2
11.0	10.6	9.3	9.3

Table 2—.Relative growth and sporulation of *Cladosporium cucumerinum* on individual amino acids in a Richard's solution with and without potassium nitrate.

	0.3 g N [a]				0.1 g N			
	Growth		Sporulation		Growth		Sporulation	
Amino acid	$-KNO_3$	$+KNO_3$	$-KNO_3$	$+KNO_3$	$-KNO_3$	$+KNO_3$	$-KNO_3$	$+KNO_3$
DL-*a*-alanine	0 [b]	+	+	+	0	+	+	+
DL-*a*-amino-n-butyric acid	–	–	+	+	0	+	+	+
L-arginine	–	–	+	–	0	0	+	+
L-*g*-amino butyric acid	0	0	0	–	+	+	0	0
L-asparagine	+	0	+	0	+	0	+	0
L-aspartic acid	+	+	+	–	+	+	+	–
L-citrulline	0	0	+	+	+	+	+	+
L-cysteine	None	None	None	None	None	None	None	None
L-cystine	–	–	–	–	–	0	–	0
L-glutamic acid	+	+	–	–	+	+	–	–
L-glycine	+	+	–	–	0	+	–	–
L-histidine · HCl · H_2O	+	+	–	–	+	+	–	–
L-leucine	–	–	–	–	–	0	–	0
L-lysine · HCl · H_2O	–	+	+	–	–	0	0	–
L-methionine	–	–	–	–	–	0	–	0
L-phenylalanine	–	–	+	–	0	0	+	–
L-proline	0	+	+	–	+	+	+	0
L-serine	0	+	–	–	0	+	–	–
DL-threonine	–	+	+	–	0	+	+	–
L-tyrosine	–	+	+	–	–	–	+	–
DL-valine	0	+	–	–	0	+	–	–
DL-isoleucine	0	–	–	+	0	+	–	+
Bacto-casamino acids	0	+	0	0	+	+	0	0
None	–	0	0	0	–	0	0	0
KNO_3 [c]	0	+	0	0	0	+	0	0

[a] Amount of nitrogen contributed either by amino acid or by KNO_3. $-KNO_3$ means lacking KNO_3.

[b] Growth and sporulation are recorded relative to that produced on the basal medium containing KNO_3 at a nitrogen concentration the same as that of the amino acids tested: (–) = less than; (0) = equal to; (+) = greater than.

[c] No amino acid used; the amount of nitrogen supplied as KNO_3 was the same as that of the amino acids tested.

were made 4 and 8 days after seeding. Colonies were observed only in media containing 10^{-4} and $10^{-5}M$ cysteine when KNO_3 was present. No growth occurred on the $10^{-3}M$ cysteine treatments, with or without KNO_3, or on 10^{-4} and $10^{-5}M$ cysteine in the absence of KNO_3.

Discussion.—The results of the temperature studies may partially explain why the disease of cucumber incited by *C. cucumerinum* is not a serious problem in the South or in eastern North Carolina. Walker (6) showed that the tissues of susceptible cucumbers are essentially resistant above 25°C. The present studies show that temperatures above 30°C are lethal to the pathogen *in vitro*. Early-summer air temperatures above 30°C for several hours are not uncommon in the South, and the temperature on exposed plant and soil surfaces would be considerably higher. Such temperatures would tend to eradicate the fungus.

In studies of the cardinal pH values for growth of microorganisms on synthetic media, the constituents of the medium, especially the buffering agents, must be selected with care. Furthermore it is essential that the components of the medium used be included in reports of pH range studies, as pointed out by Lilly and Barnett (4). The results of these tests emphasize the importance of those precautions.

The organism was shown to be capable of breaking-down and using numerous different carbohydrate and nitrogen compounds. Cysteine was the only nitrogen compound that the pathogen failed to use. All carbon sources tested were metabolized.—North Carolina State College.

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VIRUS-HOST RELATIONSHIPS IN PLANT TISSUE CULTURE

Jia-Hsi Wu, A. C. Hildebrandt, and A. J. Riker

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SUMMARY

When tissue cultures of tobacco (*Nicotiana tabacum* × *N. glutinosa*), single-cell clone (H 239) at different stages of cellular development, were inoculated with tobacco mosaic virus (TMV) young cultures (largely meristematic) were more resistant to TMV infection and/or to subsequent multiplication of TMV than were older cultures composed largely of enlarging or senescent cells.

The TMV infectivity of homogenates of a single-cell clone of tobacco (*N. tabacum*) tissue (M 222, crown gall origin) depended on how rapidly the penetrating chemicals inhibited tissue growth. A slow progressive inhibition of growth favored increase of TMV. In contrast, tissue in which growth was stopped abruptly (as when high concentrations of the antimetabolite 2,6-diaminopurine were added to the media), when assayed 4–5 weeks after inoculation, had a lower TMV infectivity than did the control. The above observations suggested that active cell division accounted for low infectivity per unit fresh weight of tissue, and it was inferred that nucleoprotein synthesis required for cell division is competitive with TMV synthesis.

A single-cell clone of tobacco tissue (M 222), when infected with a TMV preparation from field-grown infected plants, selected a mild strain of TMV when continuously subcultured *in vitro*. The TMV-infected M222 tissue divided as well as uninfected tissue. Infected tissue became brownish-yellow with age whereas uninfected tissue did not. The infected tissue also differed in growth response to uracil and its analogs added to culture media.

No mutants were obtained by growing tissues infected with a very mild, small-lesion-forming strain of TMV on media containing chloramphenicol and pyrimidine analogs including the bacteriophage mutagen 5-bromouracil.

Introduction.—The tissue-culture method has been used extensively to elucidate the nature and host relationships of animal viruses. More recently, plant viruses have been studied in plant-tissue cultures. Aucuba mosaic virus multiplication has been studied in isolated root-tip cultures (27). Tobacco stem tissue grown *in vitro* was successfully inoculated with TMV by Segretain (21), and virus-infected tissue was isolated by Morel (18), who found that the virus multiplied continuously in successive subcultures. Differences in susceptibility of different single-cell clones to infection by TMV and/or subsequent multiplication of TMV were studied by Hildebrandt (4). Nucleic

acid components (purines and pyrimidines) and their analogs were found to influence virus concentration in infected tobacco tissues (5, 11). The present study describes 1) the effect of age and type of cells on susceptibility to infection and/or subsequent multiplication of virus, 2) virus selection by a single-cell clone of tobacco tissue, and 3) the relationship between the amount of virus synthesized and the amount of growth of the host tissue that was influenced by antimetabolites. Portions of the results have been abstracted (28).

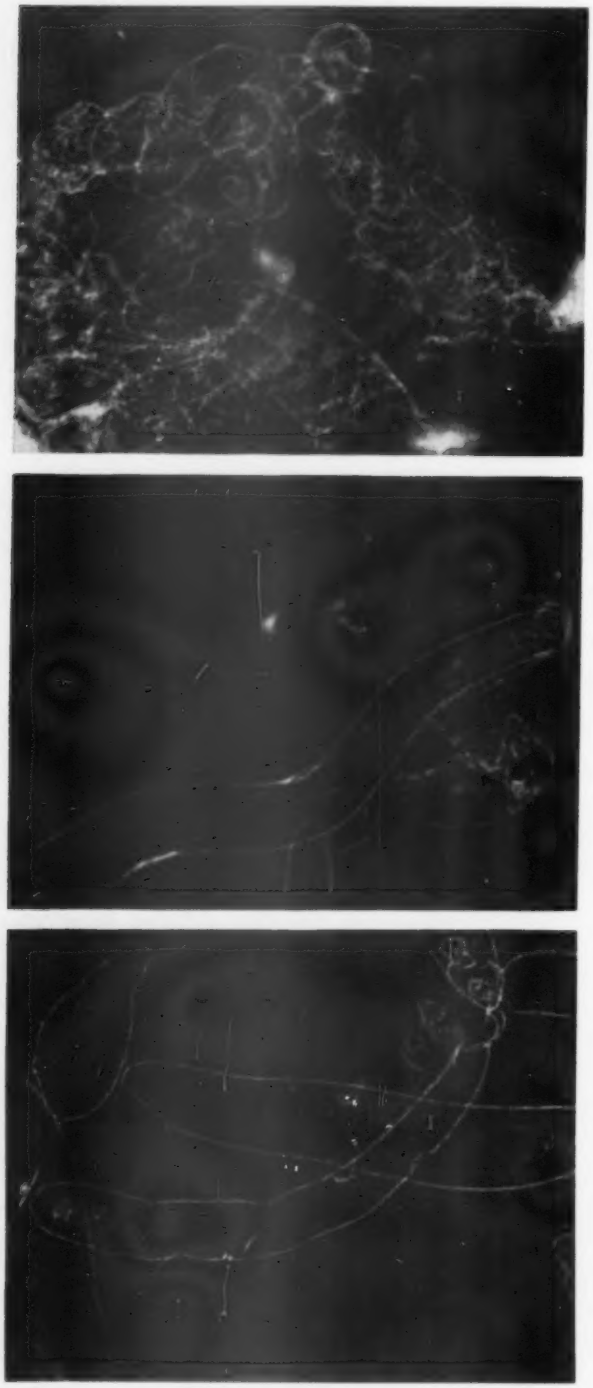

Fig. 1.—Tobacco tissue (H239) showing changes in cell types. Top: 6 days after subculture from 4-week-old culture, constituted mostly of small meristematic cells. Middle: 3 weeks after subculture, enlarging and mature cells. Bottom: 5 weeks after subculture, showing older cells.

Materials and methods.—The tobacco tissue cultures used were of single-cell origins (4), isolated according to the method of Muir et al (19) and maintained in this laboratory for some years. The single-cell clone H 239 was originally isolated from hybrid tobacco (*Nicotiana tabacum* L. × *N. glutinosa* L.), and clone M 222 was isolated from Morel's *N. tabacum*. The culture medium ("D" medium) was White's (26) medium as modified by Hildebrandt et al (6), supplemented with coconut milk (150 ml/L) and naphthaleneacetic acid (0.1 mg/L) (2), and 2,4-dichlorophenoxyacetic acid (6 mg/L) as described by Steward and Caplin (23). Liquid medium was prepared by omitting agar (0.6%) from the same medium. Prescription bottles (120 ml) were used as culture vessels (39 ml culture medium were used), and all the cultures were incubated in a dark room at 26° and at 55% relative humidity. Liquid cultures were on a reciprocating shaker (60 cycle/min.). The virus sources used for inoculations were either from field-grown tobacco infected with ordinary strains of tobacco mosaic virus (TMV) or from a purified sample of Johnson's mild strain of TMV. The tissues were incubated in the liquid shake culture and inoculated with TMV preparations filtered through a UF bacteriological sintered-glass filter. No artificial wounding with abrasives or otherwise was used to inoculate the culture with the filtered TMV.

Virus titer in the tissues was estimated by grinding the tobacco tissues diluted with 0.05*M* phosphate buffer in proper concentration and inoculating a local-lesion host plant, *N. tabacum* × *N. glutinosa*, with the homogenate. One half of each of 8 leaves was inoculated with the cultured tissue homogenate and the opposite halves with a TMV standard (purified TMV at 2 mg/ml). The relative infectivities of the homogenates of the tissues were calculated by dividing the number of lesions produced by tissue homogenate by the number of lesions produced by the TMV standard.

Experimental results.—*General cell morphology in liquid shake culture.*—The single-cell clone (H 239) of hybrid tobacco (*N. tabacum* × *N. glutinosa*) when subcultured in liquid medium, changed in cell morphology as it aged. When day-to-day observations were made after subculture of a 4-week-old tissue in new culture medium, the cell types observed as growth progressed were very much like those in the growing root tip (Fig. 1). During the first period (1–7 days after subculture), the cells were predominantly of the meristematic type. Such cells were small, contained a large nucleus, and had a relatively small vacuolar space. The cellular activity during this period was probably dominated by cell division. During the next period, the cells were largely in the enlargement and maturation phase. That period was comparable

to the elongation phase of root tips, except that the free-floating cells elongated and enlarged very much more than do those of root tips. Some giant cells reached 1–2 mm in length and could be seen by the naked eye. This period lasted about 4 weeks, during which a few tracheid elements appeared. The third phase was characterized by the senescent condition of most cells. The cell contents gradually disintegrated, and the plastids gradually became small and finally disappeared; certain cells, however, rejuvenated to produce one to many endogenous cells (9).

Susceptibility of different cell types to TMV.—It was of interest to see whether there was any relationship between the different cell types and their susceptibility to TMV infection and/or to subsequent multiplication of the virus. Therefore, tissue cultures (H 239) in liquid media were inoculated with a filtered TMV preparation (free of contaminating microorganisms) 3, 10, 17, 25, 32, or 40 days after subculture. One week after inoculation in each case, the infected tissues were transferred to a Büchner funnel and rinsed 7–8 times with phosphate buffer (0.05*M* phosphate, pH 7.0) to remove the TMV inoculum. The last washing fluid induced a few or no lesions when tested on the hybrid tobacco used for assay.

The tissues were then ground with a mortar and pestle and the resulting homogenates assayed for TMV. Although there was considerable variability in the lesion counts the results suggested that young cultures with most cells in the meristematic phase inoculated 3 days after subculture were more resistant to TMV infection and/or to subsequent multiplication than were older cultures. The older cultures inoculated after 10, 17, or 25 days consisted largely of enlarging cells, or if inoculated after 32 or 40 days, senescent cells. The greatest amount of virus was assayed in the oldest cultures inoculated 32 to 40 days after subculture.

Selection of very mild TMV strain by tissue culture.—The single-cell clone (M 222) of *N. tabacum* tissue of crown gall origin was cultured in medium and inoculated with TMV from infected field-grown tobacco plants. Subsequently, the infected tissues were subcultured on solid agar (0.6%) medium, and cultured 4–5 weeks. After 7 such subcultures, samples of tissues were homogenized and the homogenates assayed for TMV. Numerous local lesions were produced on the test plants, an indication of continuous multiplication of TMV in the tissue cultures. These local lesions, however, were different from those produced by Johnson's severe or mild strains (8), being consistently far smaller in diameter and paler in color (Fig. 2).

Homogenates from individual small lesions of the tissue-culture-selected strain were inoculated to individual leaves of tobacco plants (*N. tabacum*, variety

Fig. 2.—Lesions induced by 3 strains of tobacco mosaic virus on the hybrid tobacco leaf. Left half: Johnson's mild strain. Right half: Johnson's severe strain (large lesions), and very mild strain from tobacco tissue culture (small lesions). All inoculations were made at same time, and the picture was taken 12 days after inoculation.

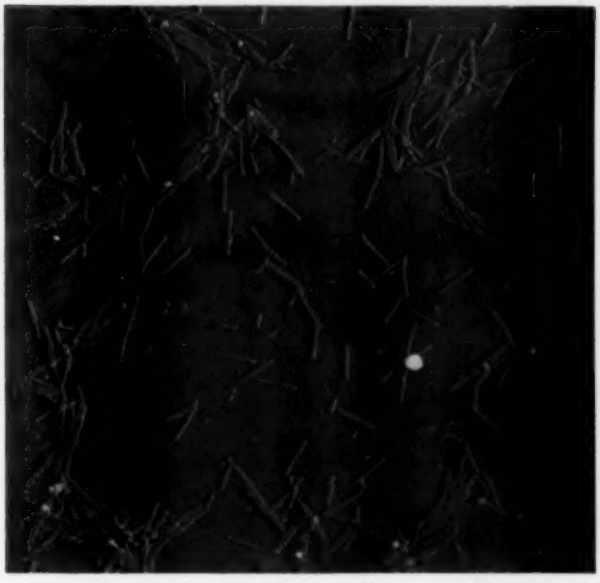

Fig. 3.—Electron micrograph of the very mild strain of TMV, showing typical rods of TMV. Approximate magnification 40,000×. Photograph from Lillian Cooper, A. C. Hildebrandt, P. J. Kaesberg, and A. J. Riker.

Table 1.—Symptoms on different host plants inoculated with Johnson's severe and mild strains and the very mild strain of TMV selected by passage through tissue cultures.[a]

Host plant	Severe strain	Mild strain	Very mild strain
N. tabacum × *N. glutinosa*	Lg. necr. les.	Md. necr. les.	Minute necr. les.
N. tabacum (Havana 38)	System. Sev. distort. 5-6 days	System. No distort. mild. 7-9 days	System. Very mild. 11-14 days
N. sylvestris	System mosaic	Large necr. les.	Small necr. les.
Lycopersicum esculentum	System. mosaic	None	None
Phaseolus vulgaris var. *pinto*	Necr. md. les.	None	None
N. longiflora			
N1-V5-4-1-2	System.	Loc. (?) system.	Loc. (?) system.
N1-f-11-1	System.	Loc. necr.	Loc. necr.
N1-f-3-2-1-2	System.	Loc. necr.	Loc. necr.
N1-V5-8-1-1	System.	Loc. necr.	Loc. necr.
Petunia			
P-6-1-1-1-2	Loc.	Loc. necr.	Loc. necr.
P17-1-3	System. mosaic	Loc. necr.	Loc. necr.

[a] Abbreviations: lg. = large; necr. = necrotic; les. = lesion; loc. = local; md. = mild; system. = systemic; distort. = distortion; sev. = severe.

N. longiflora and *Petunia* lines of plants were kindly supplied by Dr. R. W. Fulton, Department of Plant Pathology, University of Wisconsin. J. H. Jensen (7) described one minute-lesion-forming strain of TMV, as J. 104, which, however, caused yellow spot on *N. sylvestris*. The present minute-lesion-forming strain produced necrotic local lesions on *N. sylvestris*.

Havana 38). Systemic symptoms were slow to develop. Vein chlorosis first appeared in about 2 weeks, compared to 6 days and 9 days, respectively, for the first appearance on similar plants inoculated with Johnson's severe and mild strains. The tissue-culture-selected mild strain induced a much milder mosaic in Havana 38 than did either of the other strains. Electron photomicrographs of the minute-lesion-forming virus showed typical TMV rods (Fig. 3).

Comparative host range and symptom studies indicated that the minute-lesion-forming strain is similar to Johnson's mild strain except the lesions were much

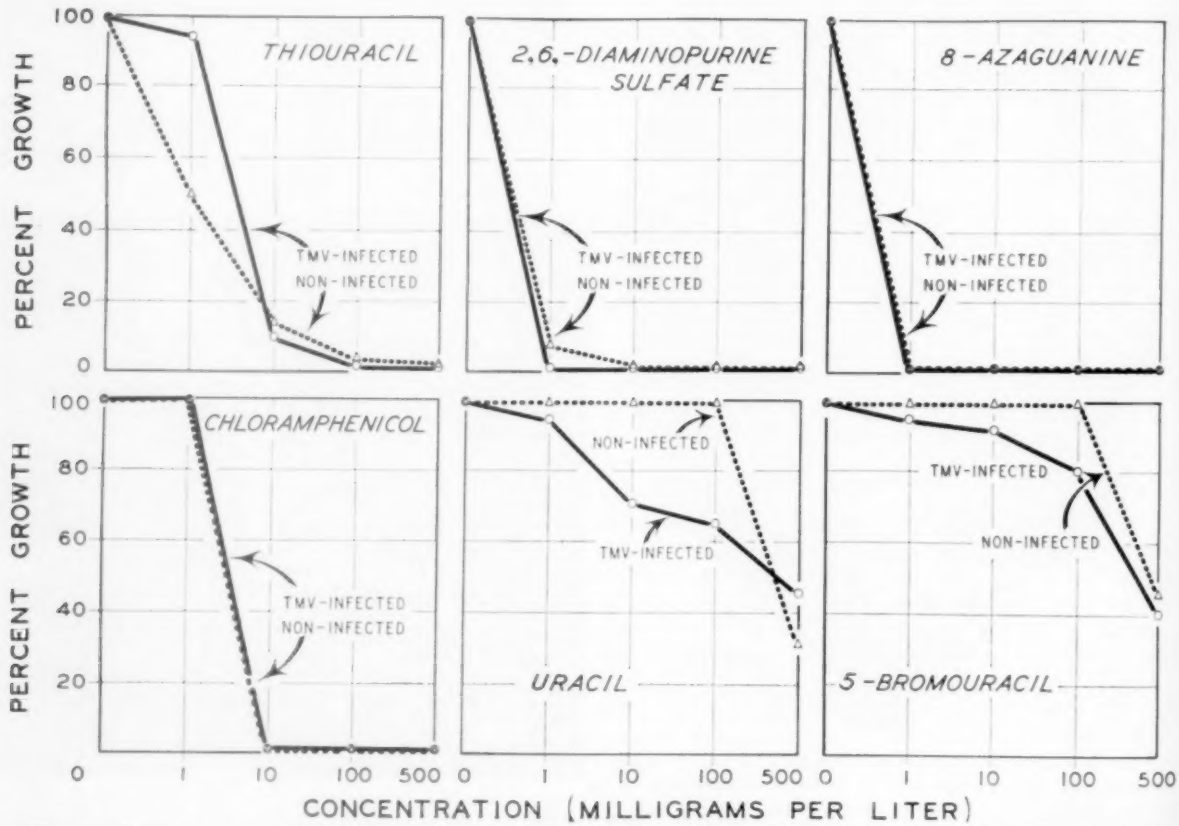

Fig. 4.—Growth response of TMV-infected and uninfected tobacco tissue (M 222) on "D" media supplemented with various concentrations of chemicals.

smaller (Table 1).

Presumably, the new strain could have arisen by mutation of TMV in the tissue culture, or it could have been present in the original inoculum and became the predominating strain because of differential selection imposed by the conditions in the tissue cultures. Since infected field plants commonly contain a mixture of strains, the original TMV inoculum was tested for the presence of very mild strains. Hybrid tobacco plants in the greenhouse were inoculated with original inoculum. Of the 6,000 lesions produced on 20 plants, 40 were used for single-lesion transfers to hybrid tobacco plants. The homogenate from one lesion produced lesions of a size similar to that of lesions produced by the tissue-culture-selected mild strain. Consequently, the above results are interpreted as the result of virus selection by a particular tissue and its nutritional environment. Analogous phenomena of changes in lesion size following frequent subculture of Coxsackie A9 virus in the carrier of HeLa cells has been reported by Takemoto and Habel (24).

Some comparative physiological studies on infected and uninfected cultures of single-cell clone M 222.—Infected tissues on the solid (0.6% agar) "D" medium commonly grew as well as did uninfected tissues. Infected tissue, however, was distinguished macroscopically from uninfected tissue because it was brownish-yellow. In tests designed to determine whether infected and uninfected tissue differ physiologically, the 2 kinds of tissue were cultivated on solid "D" media containing various concentrations of some pyrimidines, purines, or their analogs. Generally the growth responses of infected and uninfected tissues to those chemicals tested were very much alike (Fig. 4), but slight differences were noted in media containing 2,6-diaminopurine or thiouracil. Infected tissues subcultured on media containing 2,6-diaminopurine (1 μg/ml) failed to grow, whereas uninfected tissue grew very slowly. In media containing thiouracil (1 μg/ml), infected tissue grew better than did uninfected tissue.

Infectivity of tissues grown on "D" medium containing various amounts of chloramphenicol, pyrimidine, purine, or their analogs.—Subcultures of infected tissues were made by transferring an average 0.22 g of tissue of 4- to 5-week-old cultures. Growth was measured as the wet weight after a growth period of 4–5 weeks. The tissue cultures on "D" medium served as control; these grew to an average 9.5 g during the incubation period. The relative TMV infectivities of control cultures were taken as 100%.

The effect of chemicals on the infectivity of TMV in the tissue-culture homogenates varied with the chemicals added to the medium on which the TMV-infected tissues were grown and with the concentrations (Fig. 5, 6). Since thiouracil, 2,6-diaminopurine, and 8-azaguanine have been reported to be powerful inhibi-

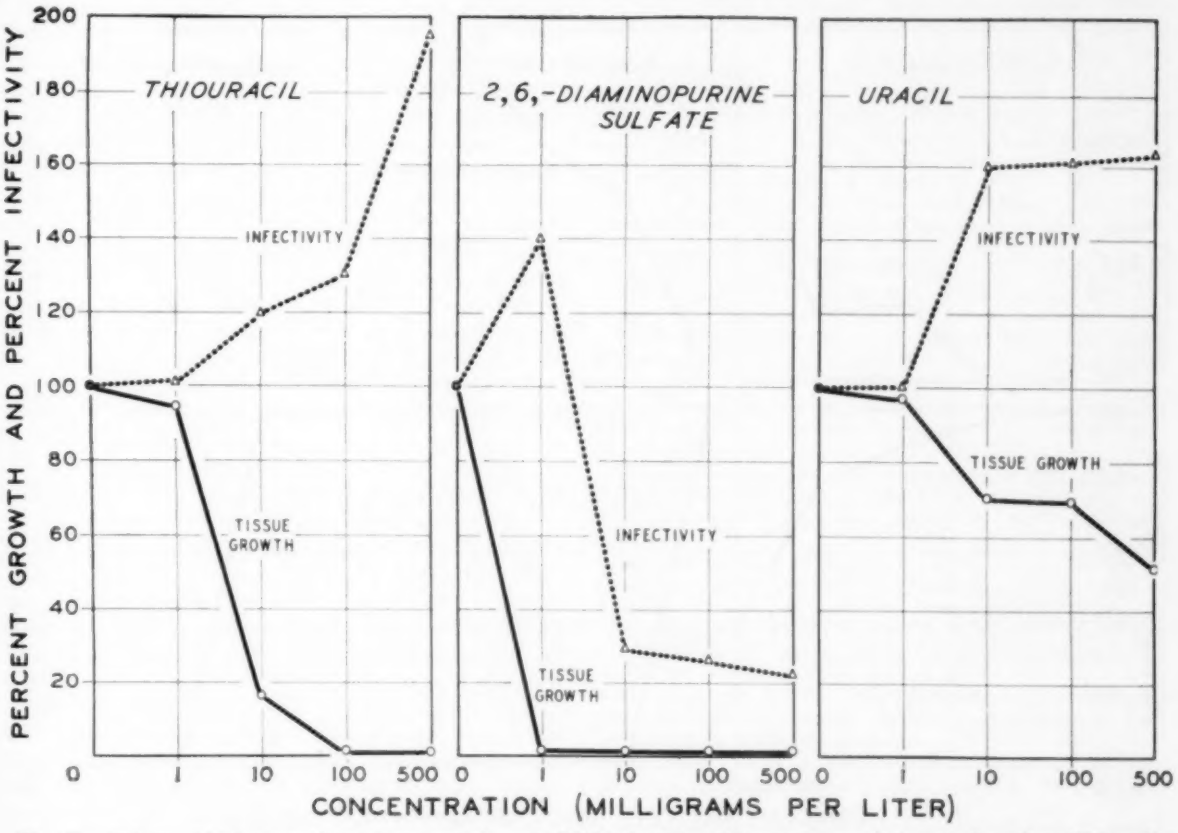

Fig. 5.—Relationship between host tissue growth and TMV infectivity of homogenates of tissue cultured on "D" media with addition of various concentrations of thiouracil, 2,6-diaminopurine sulfate, or uracil.

tors of TMV synthesis in plant leaf discs (16), it is surprising that at some concentrations these materials appeared to stimulate virus synthesis. When the medium contained 1, 10, or 100 μg of 8-azaguanine per ml, 1 μg of 2,6-diaminopurine per ml, or any of the concentrations of thiouracil used, the TMV infectivities of tissue homogenates were higher than that of the homogenates from control tissues. Only at high concentrations (10 μg/ml and higher concentrations) was 2,6-diaminopurine inhibitory. When certain concentrations of thiouracil (100 and 500 μg/ml), 8-azaguanine (1, 10, and 100 μg/ml) or chloramphenicol (10, 100, and 500 μg/ml) were used the tissues cells apparently failed to divide, but the amount of virus synthesized was 2–4 times as great as that formed in the controls. These results resemble those of Rubin and Temin (20), who reported that chick fibrioblast cells that had started to produce Rous sarcoma upon irradiation lost their ability to divide, yet continued to produce virus.

When uracil, 5-bromouracil, or chloramphenicol were used, the infectivity of homogenates from the TMV-infected tissue cultures increased when growth decreased. The infectivity per unit wet weight of tissue was highest in homogenates from tissues that grew poorly.

Chloramphenicol, a chemical used extensively in studies on bacteriophage synthesis, has been reported to specifically inhibit protein synthesis (3, 17, 25). In the present tests concentrations of 10, 100, and 500 μg/ml completely inhibited cell division of infected tobacco tissue, but TMV infectivity in the homogenates was increased as the chloramphenicol concentration was increased. Thus, chloramphenicol was not effective in inhibiting TMV nucleoprotein synthesis of TMV-infected tissue, but was effective in inhibiting cell division activity. The increase in infectivity of non-growing tissues could not be attributed to loss of water from tissues, because the average weight of the seed piece, which was originally about 0.22 g, remained fairly constant (about 0.21–0.18 g at the end of incubation period) on 0.6% agar culture medium.

The effect of 5-bromouracil was of interest because of its mutagenic effect on T_2 bacteriophage (12, 13). The infected tissues in this experiment contained the very mild strain of TMV, which produces very small necrotic local lesions on hybrid tobacco leaves. Hence, any mutation from small lesions to larger lesions would have been detected. No such mutants were detected. Siegel (22) reported that the mutation changes on lesion size were unidirectional—from large to small lesions. This might be the reason that no mutants were obtained.

Discussion.—TMV is a nucleoprotein that by itself possesses no independent metabolic system; therefore, the infection and multiplication of TMV is dependent

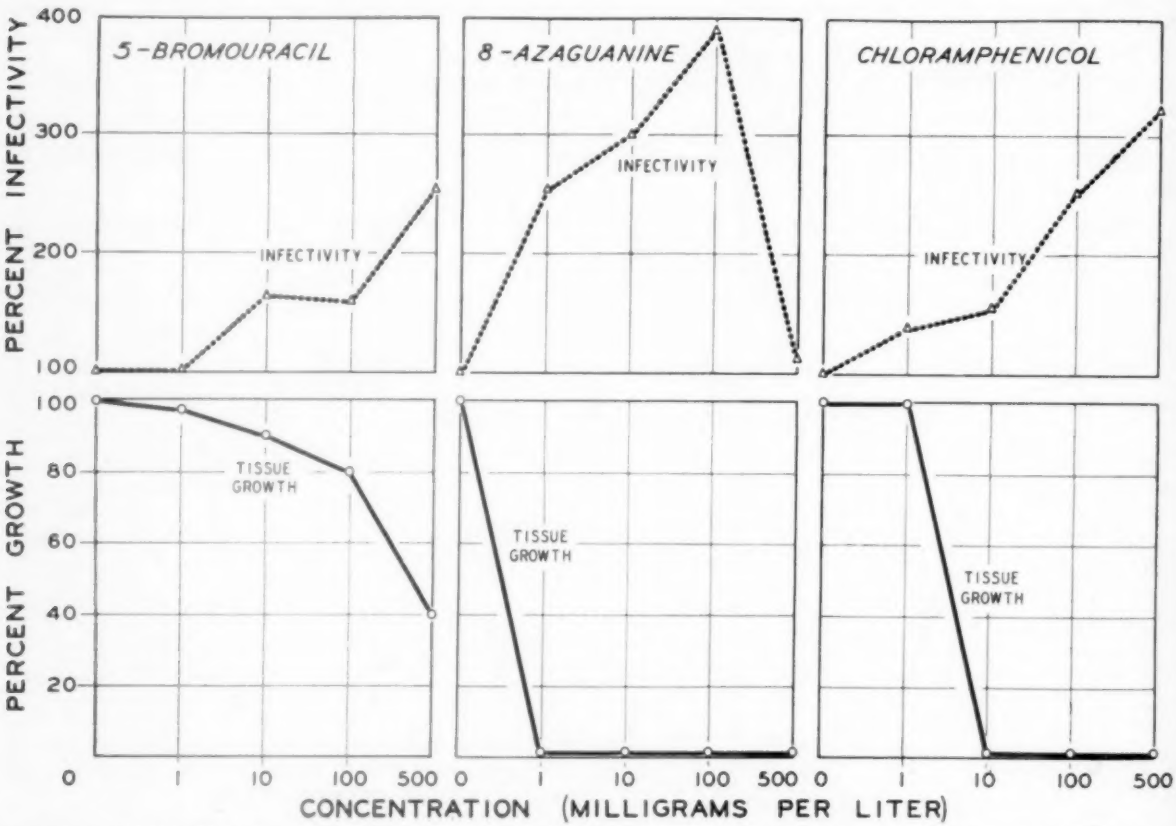

Fig. 6.—Relationship between host tissue growth and TMV infectivity of homogenates of tissue cultured on "D" media with addition of various concentrations of 5-bromouracil, 8-a zaguanine, or chloramphenicol.

on the host metabolism. Accordingly, studies of biosynthesis of TMV and its relation to host physiology are fundamental. The results of the present experiments indicate that the amount of virus synthesized per unit weight of tissue depends on host physiology.

There was an apparent relation between the predominant cell type and susceptibility to virus infection and/or to subsequent virus multiplication. Generally, the meristematic cell type was more resistant to TMV infection and/or subsequent virus multiplication than were more mature types. It is possible that the relatively low concentration of virus in the meristematic region of roots is related to faster growth (cell division) of meristematic region than of the movement of virus from cell to cell (10), but it could also be due to the inherent resistant nature of the meristematic cells to TMV infection and/or subsequent multiplication.

Thiouracil, 8-azaguanine, and 2,6-diaminopurine have been regarded as powerful inhibitors of TMV multiplication (16) in leaf tissues. When these chemicals were added to the culture media and TMV-infected tissues were cultured on the media, however, the TMV infectivity of the tissue homogenates was not necessarily lower than that of control tissues subcultured on the same media without addition of the chemicals: infectivity of the tissue homogenates depended on the physiological growth conditions. Thus, in the case of media supplemented with thiouracil, little or no growth of the tissue (fewer cell divisions) favored increased infectivity per unit wet weight. When certain concentrations of 8-azaguanine or 2,6-diaminopurine were added, the tissue failed to grow but supported more TMV synthesis than did the control tissues. Infectivity was low when higher concentrations of 2,6-aminopurine or 8-azaguanine were added to the media. It appears that the final infectivity of tissue homogenates depended on how rapidly the chemicals inhibited normal cell division. When cell division was slowed gradually, without killing the tissue in short time, infectivity increased. This relation was also indicated when uracil, 5-bromouracil, or chloramphenicol was used. The above observations generally agree with Augier De Montegremier and Morel's (1) conclusion that the concentration of virus is diminished by rapid tissue proliferation.

It is further inferred that the synthetic activities involved in TMV multiplication are competitive with those involved in cell division. Although both involve nucleoprotein synthesis, the results suggest that distinctly different pathways of synthesis are involved. With this hypothesis, the low infectivity of meristematic tissue could be explained on the basis of a strong competition between TMV nucleoprotein synthesis and synthesis of nucleoproteins essential to cell division.

Although meristematic cells are relatively resistant to TMV infection and/or subsequent virus multiplication, the experiments with single-cell clones of tobacco tissue (M 222) infected with the very mild strain of TMV indicated that TMV-infected cells divided on "D" medium because the infected tissue culture grew as well as uninfected tissue.

The yellow-brownish coloration of infected cells suggested another difference in the physiological aspect of infected cells. The coloration may result from an accumulation of polyphenolic compounds (15), resulting from an increase in polyphenoloxidase activity, as reported by Martin (14). Growth response of infected and uninfected tissue to low concentration of uracil, thiouracil, 5-bromouracil, and 2,6-diaminopurine added in "D" media also suggested some physiological differences between the two tissues.—Department of Plant Pathology, University of Wisconsin, Madison 6.

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INHIBITION OF FUNGI BY FILIPIN AND ITS ANTAGONISM BY STEROLS

David Gottlieb, H. E. Carter, Lung-chi Wu, and J. H. Sloneker

Respectively, Department of Plant Pathology and Department of Chemistry and Chemical Engineering, University of Illinois; Department of Botany, Michigan State University; and Northern Utilization Research and Development Division, U.S.D.A., Peoria, Illinois.

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SUMMARY

Filipin prevents spore germination of *Aspergillus flavus* and other fungi as well as inhibiting their growth. At low concentrations, the antibiotic causes the formation by *A. flavus* of short-celled, branched germ tubes. In the presence of the antibiotic, swellings appear on the mycelial filaments and subsequently burst with loss of protoplasm. Yeasts tend to produce mycelial growth in sublethal concentration of the antibiotic; higher levels appear to cause granulation of protoplasm, irregular cell walls, and clumping of the cells. Seventy-seven fungi representing 46 genera and 60 species were inhibited by the antibiotic. The addition of cholesterol to the medium prevented the antifungal effect of filipin on 31 organisms, but not all polyenic fungicides are thus affected. Active sterols often had a long chain on the phenanthrene nucleus; steroids were inactive except for androstanol-3β-one-17. Various modifications of the cholesterol molecule allow the antagonistic effect on growth inhibition of *Saccharomyces cerevisiae* by filipin, but others prevent this activity. Adding the antibiotic at the time of inoculation of the medium lengthens the lag phase of growth. No correlation exists between the sterol content of a fungus and its sensitivity to the antibiotic. Similarly, prior growth on cholesterol-containing medium does not change the sensitivity of *S. cerevisiae* to the antibiotic. The yeast does not readily acquire resistance to the antibiotic in shake culture. Cysteine and reduced glutathione will also prevent the antibiotic action of filipin.

Previous papers in this series have described the isolation, purification, and properties of the antifungal agent filipin (3, 10, 25), one of the polyene antibiotics having 5 conjugated double bonds. It is a crystalline, water-insoluble compound with a molecular weight of 571, and exhibits the triple peak ultraviolet-light absorption characteristic of such compounds. The antibiotic inhibits the growth of fungi, but not that of any of the bacteria thus far tested. It is toxic to some higher plants as well as animals, and to at least one protozoan species. We recently called attention (11, 12) to a unique relationship between filipin and some sterols: that many of the naturally occurring sterols, such as ergosterol, cholesterol, sitosterols, and stigmasterol, prevent the toxic action of filipin against fungi. Further investigations of these phenomena are reported herein.

Sterols are very widely distributed among living organisms and have been found in most of the animals and plants studied (4, 5, 6, 7, 24). Very few instances of sterols in bacteria are known, and of those investigated, only *Azotobacter chrococcum* and *Escherichia coli* contain sterols in detectable amounts (7). Ergosterol is the predominant sterol in both the yeast and filamentous fungi. Yeasts also contain many other sterols; thus various isolates of *Saccharomyces cerevisiae* also have zymosterol, ascosterol, fecosterol, episterol, anasterol, hyposterol, cerevisterol, 5-dihydroergosterol, tetraethanoid sterol, and Wieland's sterol (8, 9). Filamentous fungi have yielded 14-dehydroergosterol, fungisterol, stigmasterol, and mycosterol, as well as the ubiquitous ergosterol (7, 8). Cholesterol (Fig. 1), the best-known sterol of animals, is distributed widely, from lipids of marine invertebrates to man. Stigmasterol and sitosterol are the best-known sterols of higher plants (5).

The location of sterols in the cell may vary with the species. In *Neurospora crassa* they are associated with the cellular residues (20). So too in *Torulopsis lipofera*, the cellular debris fraction contains more ergosterol than the other cellular fractions (16). The greatest concentrations of ergosterol in *S. cerevisiae* are associated with the larger particle fractions (15). The biosynthetic pathway of ergosterol formation in yeast is being elucidated, and has been shown to take place with acetate as a building unit through mevalonic acid and squalene (2, 13, 14, 18, 19, 22).

Because of the possible involvement of sterols in

the mechanism of inhibition of fungi by filipin, a knowledge of some of the factors concerned in the inhibition of fungi by the antibiotic, as well as the antagonistic action of sterols, is vital to clarification of this phenomenon.

Materials and methods.—Difco dehydrated yeast nitrogen base (YNDB) with 1% dextrose added (YNBD) was used as the basic medium, unless otherwise indicated. The medium was prepared with 6.7 g yeast nitrogen base, 10 g dextrose, and 1000 ml of distilled water; when semisolid medium was needed, 20 g Difco bacto agar was added. Cholesterol was Eastman Organic Chemical Co. reagent grade; Eastman reagent grade ergosterol was recrystallized before use; the other sterols were crystalline compounds obtained from different sources. Where purity was in doubt, melting points were determined. Reagent-grade methanol or ethanol was used to dissolve both the sterols and filipin.

Inocula for most experiments were obtained by growing (mycelial) fungi for 5 days at 26°C, on potato-dextrose agar slants, and the yeasts for 3 days on YNBD agar slants. Ten ml of distilled water containing 10 ppm of Tween 80 were added to each slant, and the spores or yeast cells were removed by gently rubbing the surface of the culture with a bacteriological transfer loop.

Plates for studies of the antibiotic spectrum of filipin were made by dissolving the antibiotic in methanol and adding the solution to sterile Difco Czapek Dox Agar to give concentrations of 0, 5, 10, and 20 μg/ml. The inoculum was streaked on the agar plates and incubated at 26°C. Growth of the fungi was recorded at 2. 3, and 6 days. Preliminary studies on protection by cholesterol were made on double gradient plates prepared in the manner described by Szybalski (21).

Saccharomyces cerevisiae F-1, isolated from Fleischmann's baker's yeast, was used in all experiments unless other yeasts are indicated (yeasts with the designation "My" were obtained through the courtesy of Merck and Company). Inocula for experiments with yeast were prepared in one of two general ways: A) three-day-old cultures on YNBD agar slants were transferred to YNBD broth tubes to make a suspension of 10% transmission when measured on a Lumetron Colorimeter with a 530 mμ filter. The inocula were added to experimental media to give a 1% inoculum by volume, and all cultures were incubated at 26°C. B) The yeast was transferred from a YNBD agar slant to 5-ml YNBD broth in 16-mm-diameter test tubes and placed on a rotary shaker for 24 hours. The yeast was retransferred twice more to fresh tubes of the same broth to stabilize the inoculum for precise turbidometric measurements. After 24 hours on the rotary shaker, the inoculum in a 16-mm tube measured 4% transmission on the Lumetron using a 530 mμ filter. The experimental tubes were inoculated with 0.05 ml of this suspension and placed on a rotary shaker, and turbidometric measurements made at desired intervals.

Since both filipin and the sterols are insoluble in water, these chemicals were added to the media in organic solvents, filipin in absolute methanol, and the sterols in 95% ethanol. The few sterols and steroids that did not dissolve in these solvents were dissolved in acetone or chloroform. In all cases, the maximum concentration of the solvent was 1%, and solvent controls were used. Except in experiments where the effect of filipin on growing cells or the reversal of its inhibition was studied, both the antibiotic and the sterol were added immediately before inoculation of the media. All sterols or steroids that were tested for antagonism to filipin were used at a sterol-to-filipin ratio of 4:1. The results with many such compounds were verified with *Hansenula subpelliculosa* in addition to *S. cerevisiae*. Results were comparable with both organisms. All experiments were made in triplicate.

Studies with filipin are sometimes difficult because the antibiotic contains 5 conjugated double bonds and is unstable in the presence of light and air (25). During an experiment, then, the antibiotic might be inactivated below inhibitory levels if inhibitory concentrations are only minimal. It was important to determine the stability of the antibiotic under the experimental conditions generally used in these researches and whether the growth of fungi under prolonged exposure to the antibiotic might be due to inactivation of the filipin. Different concentrations of filipin in the YNBD medium, from 1 to 10 μg/ml, were inoculated with *H. subpelliculosa* and shaken on a rotary shaker for various periods up to 120 hours. Media containing 1 μg/ml filipin that were shaken for 24 hours and then inoculated, did not completely inhibit *H. subpelliculosa*, and allowed growth to 81% of the control level. This same concentration was able to inhibit growth completely for 24 hours when inoculation of the yeast and addition of the antibiotic were simultaneous. In contrast, 2 μg/ml, shaken for 96 hours and then inoculated, still retained enough antibiotic activity that growth of the yeast was inhibited completely.

Experimental results.—*Morphological effects of fili-*

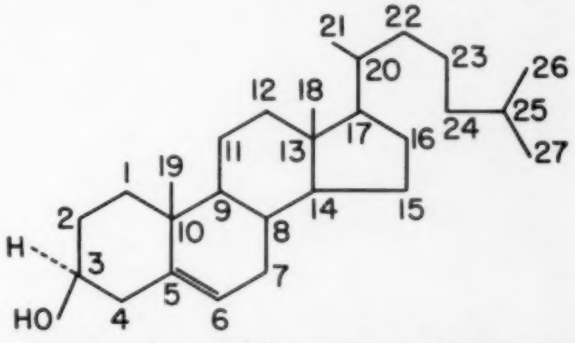

Fig. 1.—Skeleton of cholesterol (9). Carbon atoms are numbered 1 to 27. Each angle represents a carbon atom with the hydrogens needed to complete the unused valences. The open ends of short lines indicate methyl groups. Position of the double bond is denoted by Δ, with a superscript indicating the lower-numbered carbon atom of the bond. The stereomeric configuration of substituents is indicated in the skeleton by a broken line for α positions—those in which it is below the plane of the ring—or by a solid line for β positions—when the substituent is above the plane of the ring.

pin.—Filipin produces morphological changes in yeast and filamentous fungi. When *S. cerevisiae* was incubated in 5 ml YNBD medium at concentrations of filipin of 0, 2.5, 5.0, 10, 20, and 40 μg/ml, mycelial growth was abnormal at 2.5 and 5.0 μg/ml (Fig. 2). No growth took place at the higher concentrations, but the cells became clumped, granulated, and irregular. Cells were also treated with filipin under non-growing conditions. Fleischmann's baker's yeast was washed 2 times with distilled water and then with a 0.1*M* citrate buffer. The yeasts were resuspended in the citrate buffer and placed in 500-ml Erlenmeyer flasks. Three treatments were made with filipin and cholesterol added in the following respective concentrations (μg/ml): 0 and 50, 50 and 0, and 50 and 50. The yeasts were then incubated for 20 hours at 26°C. In the treatment with filipin alone, the cells were packed together, appeared shriveled, and contained only some large granules in the interior (Fig. 2). The cells generally appeared normal in solutions containing both filipin and cholesterol.

The most drastic effect of the antibiotic on mycelial organisms could be seen with *Aspergillus flavus* grown on YNBD agar containing filipin placed on cover slips inverted over Van Teigham cells. Cells grown 48 hours in 10 μg/ml had swollen areas on the mycelium and broken sections of hyphae where some of the swellings had broken and allowed the cellular contents to diffuse out (Fig. 3-C,D). No morphological effect was observed on the spore itself when concentrations were sufficient to prevent germination. On the other hand, if spores were incubated for 10 hours in the YNBD broth medium as shaken cultures and filipin added at 5 μg/ml for an additional 5 hours, some further extension of the germ tube occurred but the cells were abnormal, short, gnarled, and branched (Fig. 3-B).

Antibiotic spectrum.—An extension of studies of the inhibitory effects of filipin to 77 cultures indicates that

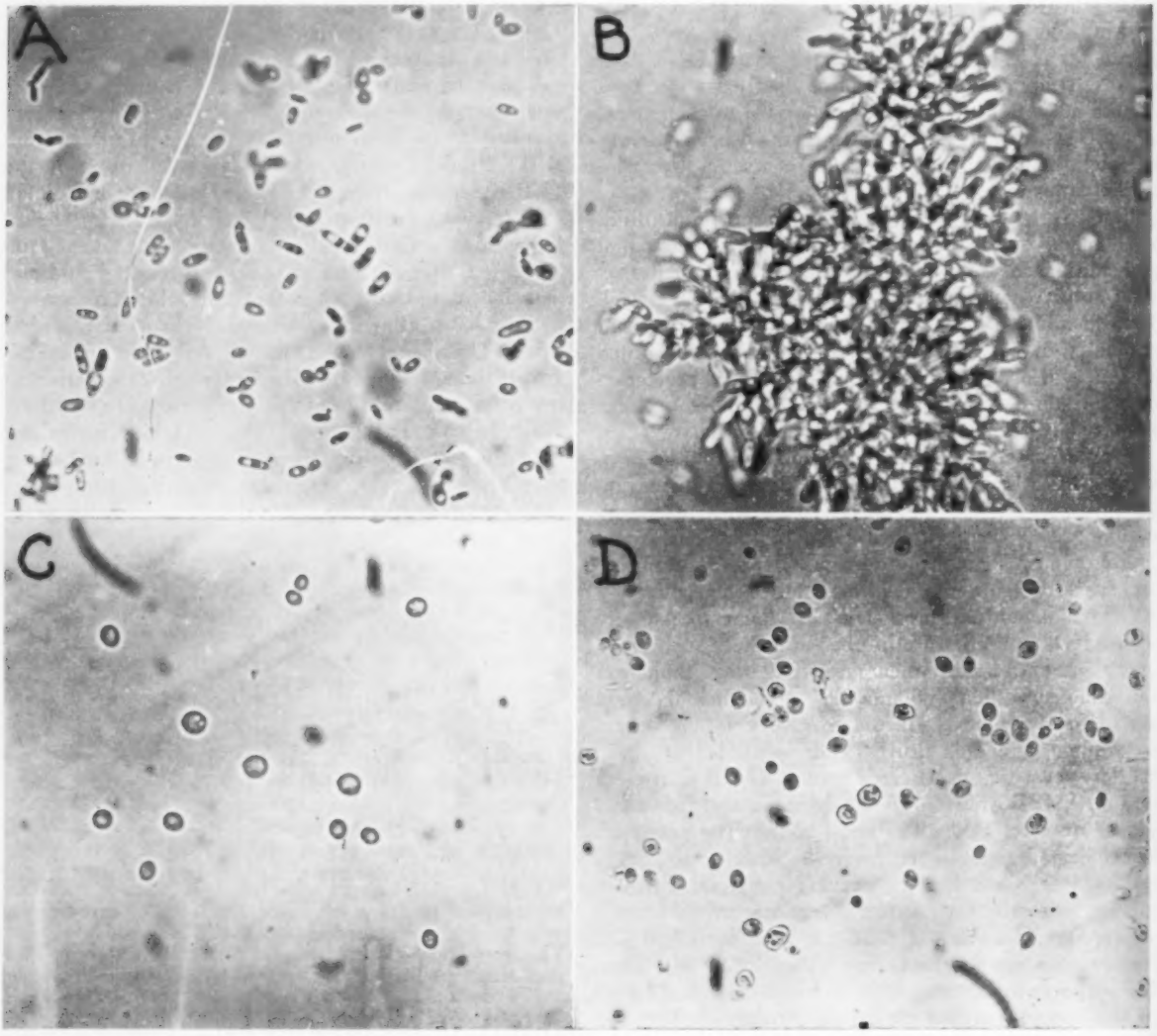

Fig. 2.—Action of filipin on *Saccharomyces cerevisiae*. **A)** Normal growth in YNBD medium. **B)** Mycelial growth in YNBD medium containing filipin at 5 μg/ml. **C)** *S. cerevisiae* suspended in 0.1*M* citrate buffer (pH, 6.0) for 20 hours. **D)** In same medium as C but containing 50 μg/ml filipin.

relatively few organisms can withstand its toxic effect. The test organisms were distributed among 46 genera and 60 species; they represented all classes of fungi and included saprophytes, plant pathogens, animal pathogens, yeasts, and filamentous fungi. Within the range of concentrations of filipin used, the sensitivity of the organisms varied from 5 to greater than 20 μg/ml of medium (Table 1). All the Basidiomycetes were inhibited by 5 μg/ml. Among the Fungi Imperfecti, species of *Aspergillus*, *Fusarium*, and *Rhizoctonia* are relatively resistant. Variations in this characteristic are great among strains of these species. Different isolates of *S. cerevisiae* needed from 5 to 20 μg/ml to prevent growth. With the more resistant microbes, which required a greater concentration of filipin for inhibition, growth sometimes occured after long periods of incubation. Part of this effect might be explained by the relative lability of the antibiotic to oxidation. Table 1 gives a partial list of the organisms studied and the inhibitory concentrations.

Differences between the sensitivity of various fungi to the antibiotic are also apparent when germination is measured. Filipin concentrations of 2.5 μg/ml were sufficient to inhibit germination of *A. niger* and *P. oxalicum* for 48 hours, and 5.0 μg/ml for 168 hours. In contrast, *A. flavus* germinated after 48 hours even in 20 μg/ml.

The concentration of filipin required to inhibit germination is greater in a shake culture than stationary culture. Spores of *A. flavus*, *A. niger*, and *P. oxalicum* were inhibited for 16 hours by 5 μg/ml YNBD medium in shake culture, but only 2.5 μg/ml were needed for 16-hour inhibition when the spores were grown in Petri plates.

Range of protection.—That the antagonism of cholesterol to filipin is a general phenomenon for many fungi

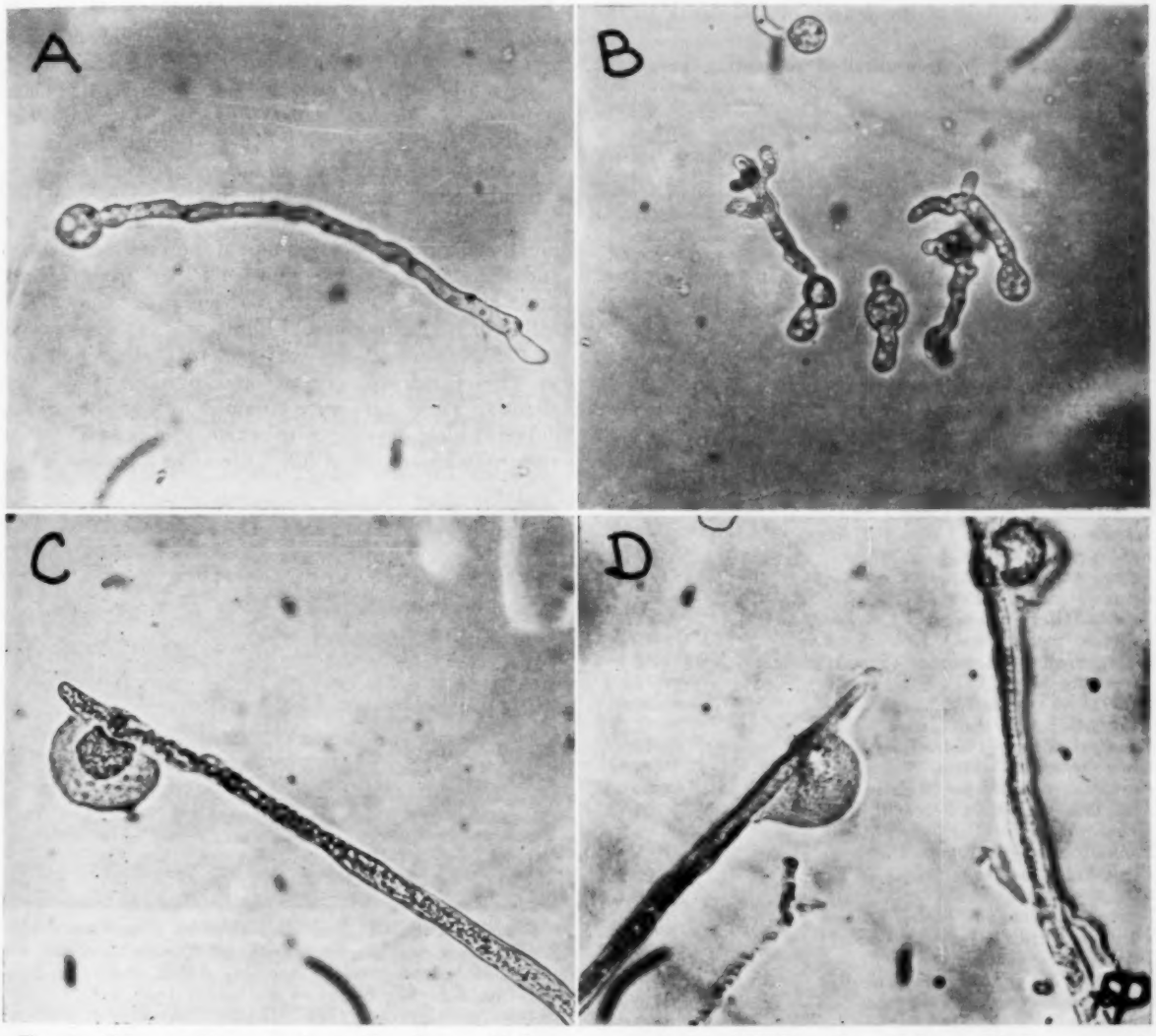

Fig. 3.—Effect of filipin on *Aspergillus flavus*. **A)** Spores germinated in YNBD medium 15 hours. **B)** Spores germinated in YNBD medium for 10 hours, and filipin added at final concentration of 5 μg/ml and incubation allowed to proceed for 5 more hours. **C,D)** Growth after 48 hours in medium containing filipin at 10 μg/ml.

is shown by the increased growth in the presence of the sterol of all the fungi that were inhibited by the antibiotic. Since cholesterol alone does not stimulate the growth of fungi, the increased growth of fungi when cholesterol is used with filipin compared with growth in filipin alone indicates a specific antagonistic effect of the sterol to the antibiotic. Double gradient plates were prepared containing filipin in the upper layer at 0, 20, and 40 μg/ml of YNBD medium; the lower layer contained the medium alone. A similar series of plates contained the same concentrations of antibiotic in the upper layer, but cholesterol had been added to the lower layer to give a concentration of 40 μg/ml. Thirty-one fungi were streaked on both sets of plates and incubated for 5 days. These fungi could be divided into three groups: 1) those that did not grow in the presence of the antibiotic, but grew completely and vigorously over the entire length of the streak when cholesterol was also present; 2) those that grew only for part of the length of the streak in the presence of the antibiotic and had complete growth with the sterol; and 3) those relatively resistant fungi that, except for a tapering toward the area of greatest concentration, and a light growth, grew the entire length of the streak even in the presence of the antibiotic, but produced a normal vigorous straight growth in the presence of the antagonist. Typical reactions of 12 fungi are shown in Fig. 4.

Table 1.—Filipin concentration preventing growth of fungi.[a]

Organism	Filipin concentration[b] μg/ml
Pythium debaryanum Hesse	>20
Mucor hiemalis Wehm.	20
Phycomyces nitens (Ag.) Kze.	5
Zygorhynchus moelleri Vuillemin	>20
Hansenula subpelliculosa Bedford	20
Saccharomyces cerevisiae Meyen	10
Saccharomyces cerevisiae Meyen	5
Saccharomyces cerevisiae Meyen	20
Saccharomyces cerevisiae Meyen	>20
Saccharomyces globosus Ostern.	5
Saccharomyces pastorianus Hans.	10
Zygosaccharomyces prioranus Klöcker	10
Monascus purpureus Went.	5
Gibberella zeae (Schw.) Petch.	5
Glomerella cingulata (Ston.) Spauld & Schrenk	10
Monilinia fructicola (Wint.) Honey	5
Exidia recisa Fr.	5
Fomes annosus (Fr.) Cke.	5
Lenzites trabea (Pers.) Fr.	5
Schizophyllum commune Fr.	5
Diplodia zeae (Schw.) Lev.	5
Colletotrichum phomoides (Sacc.) Chester	5
Alternaria solani (Ell. & G. Martin) Son.	5
Aspergillus flavus Lk.	>20
Aspergillus niger van Tiegh	5
Candida albicans (Robin) Berkh.	10
Cercospora sojina Hara	5
Fusarium moniliforme Sheldon	>20
Fusarium oxysporum Schlecht. f. *lycopersici* Sacc.	20
Helminthosporium carbonum Ullst.	5
Penicillium chrysogenum Currie & Thom	5
Penicillium oxalicum Currie & Thom	20
Stemphylium sarcinaforme (Cav.) Wilts.	10
Stemphylium solani Weber	5
Torulopsis utilis (Henneb) Ladder	20
Trichoderma viride Pers. & Fr.	5
Trichophyton interdigitale Priestley	10
Verticillium Nees & Wallr. sp.	10
Rhizoctonia DC & Fr. sp.	20

[a] Growth on Czapek Dox Agar after 6 days.

[b] Concentrations of 0, 5, 10, and 20 μg/ml were used and recorded 6 days after incubation.

A measure of the cholesterol-to-filipin ratio that would protect fungi against inhibition by filipin was studied with 11 fungi. A series of dilution plates with YNBD medium were prepared so that final concentrations of cholesterol were 0 and 10 μg/ml and those of filipin were 0, 10, 20, and 40 μg/ml. These concentrations produced ratios of cholesterol to filipin of 1/1, 1/2, 1/3, and 1/4. Growth of the fungi indicated that the organisms more resistant to the antibiotic required a higher cholesterol-to-filipin ratio to vitiate the effect of the antibiotic. The sensitive fungi, such as *Aspergillus niger, Fusarium oxysporum, Hansenula subpelliculosa, Saccharomyces cerevisiae* (My-306), *S. cerevisiae* (My-799), *S. globosus,* and *S. pastorianus,* were inhibited at a ratio of 1/4, whereas the more resistant *A. flavus, Aspergillus* sp., *Zygorhynchus moelleri,* and *S. cerevisiae* (F-1) needed 1/3 ratios.

Antagonism of sterols to other polyenes.—A preliminary study had indicated that a mixture of sitosterols obtained from carrots would protect against the toxicity of a wide range of polyenic antibiotics and that the protection afforded *P. oxalicum* against these agents varied with the particular antibiotic. The action of fungichromin and filipin was entirely vitiated in the presence of the carrot sterols, other polyenes to a less marked degree, and nystatin only very slightly. These data were obtained by a modified agar diffusion-plate technique in which spores of *P. oxalicum* were incorporated into agar as in a regular assay

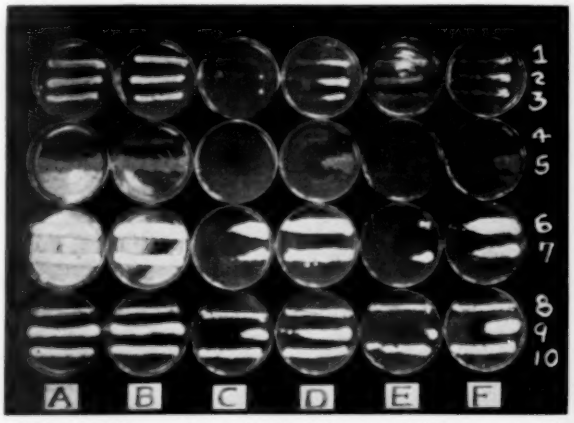

Fig. 4.—Prevention of inhibition by filipin on double gradient plates. Concentrations of filipin-cholesterol are the same in each vertical column: A-0/0, B-0/40, C-20/0, D-20/40, F-40/40. Fungi in horizontal rows are: 1) *Saccharomyces cerevisiae* (My-797); 2) *Hansenula subpelliculosa;* 3) *S. cerevisiae* (Y-132); 4) *Helminthosporium carbonum;* 5) *Phycomyces nitens;* 6) *A. flavus* (484); 7) *Aspergillus flavus* (625); 8) *Zygorhynchus moelleri;* 9) *Fusarium moniliforme;* 10) *Aspergillus* sp.

technique (23). Each polyene was added to the discs in three concentrations sufficient to produce inhibition rings of about 25 mm and less. Similar plates were prepared except that various dilutions of the carrot extract were added to each concentration of the different antibiotic solutions. Reductions of inhibition zone diameter or complete disappearance of the zone resulted. An arbitrary antifilipin unit was defined as the micrograms of filipin that would be reversed by 1 ml of the solution of carrot sitosterol. Thus, the sitosterol solution contained 128 antifilipin units per ml. From the original concentration-inhibition zone size curves for each polyene, and the reduction of the zone sizes in the presence of the sterol solution, were calculated the μg of each polyene that were antagonized per antifilipin unit. The following values were obtained (where a value of 1.0 indicates complete prevention of inhibition): fungichromin, 1.0; filipin, 1.0; amphotericin B, 0.49; trichomycin, 0.39; rimocidin, 0.39; ascosin, 0.37; candicidin A, 0.31; candicidin B, 0.19; nystatin, 0.18.

When the same polyenes were tested for the degree of protection conferred by cholesterol on *S. cerevisiae* grown in YNBD medium in a shake tube culture, the sterol protected the yeast only against filipin and fungichromin, the two most closely related antibiotics, both of the pentaene series. The ratio of cholesterol to antibiotic that would protect the yeast against 5 μg/ml of antibiotic was 2/5 for filipin and 4/5 for fungichromin. No noticeable protection by cholesterol against the other antibiotics occurred even at a ratio of 40/5. The action of two other fungistatic agents, Hyamine 1622 [*N*-di-isobutylphenoxyethoxyethyl-*N*,*N*-dimethyl-*N*-benzylammonium chloride (Hyamine 620)] and Rohm & Haas 0-963 (*N*-p-chlorobenzyl-*N*,*N*-dimethyl-*N*-t-octylphenoxyethylammonium chloride), both quaternary ammonium compounds (furnished by courtesy of the Rohm and Haas Company), was not prevented by cholesterol. The inhibition of yeast by sodium laurylsulfonate, on the other hand, was reversed by cholesterol.

Sterol structure and antifilipin activity.—A study of the antifilipin activity of 51 different sterols or steroids confirms the original observation that long-chain sterols are generally most effective in vitiating the antibiotic effect on *S. cerevisiae* (Table 2). However, no facile explanation based on molecular structure is possible at this time. The experiments were carried out in shake culture according to the general procedures described, and growth was measured turbidimetrically. The concentration of filipin used was 2 μg/ml, and the sterols or steroids were always used at a concentration of sterol to filipin of 4/1, and generally also 2/1. The higher concentration is 16 times as great as the ratio required to prevent antibiotic activity by cholesterol.

The outstanding feature of the study was that all but one of the active molecules contained the long aliphatic side chain in compounds from C_{27} to C_{29}. The exception was androstanol-3β-one-17, which is a C_{19} compound. More analogs of cholesterol (Table 2) than of any other compound were available, and data from the action of these materials indicate that a considerable variation in the molecule is possible without loss of antagonistic property. Some of these are listed below:

Table 2.—Antagonism af steroids and sterols to inhibition of *Saccharomyces cerevisiae* by filipin.[a]

Compound[b,c]	No. of carbon atoms	Growth[d]
Estrone [1]	18	—
Estradiol [1]	18	—
Androsterone (androstanol-(3α)-on(17) [1]	19	—
Androstanol-(3β)-on-(17) [1]	19	+
Androstandiol-(3β,17β) [1]	19	—
Δ^2-androstenon-(17) [1]	19	—
Androstendione [1]	19	—
Δ^5-androstenol-(3β)-on-(17)-acetate [1]	21	—
Testosterone [1]	19	—
17-methyl testosterone [1]	20	—
Cortisone [1]	21	—
Desoxycortisone [1]	21	—
Hydrocortisone [1]	21	—
11-desoxy-17-hydroxy corticosterone [1]	21	—
11-desoxy-17-hydroxy corticosterone [1]	21	—
Pregnandiol-(3α, 20α) [1]	21	—
Δ5-pregnenol-(3β)-on-(20) [1]	21	—
Allo-pregnanol-(3β)-on-(20) [1]	21	—
Progesterone [1]	21	—
Dehydronorcholen [1]	22	—
Iso-dehydronorcholen [1]	22	—
$\alpha\Delta^5$-17α-allyl-androstendiol-(3β,17β) [1]	22	—
Cholic acid [1]	24	—
Desoxycholic acid [1]	24	—
12-ketocholic acid [1]	24	—
Cholesterol [1]	27	+
Cholestanol (3β)-(ol) [1]	27	+
Cholestenone [1]	27	+
$\Delta^{3,5}$-cholestadiene [1]	27	+
Δ^5-cholestene-3-COOH [3]	28	±
Cholestanon-3 [2]	27	+
Cholesteryl acetate [1]	29	+
α-cholesterinoxide [1]	27	+
Cholestandion-(3,6)-ol-(5) [1]	27	+
Δ^4-cholestene-3-one [2]	27	±
Δ^4-cholestenedione (3,6) [1]	27	±
Δ^4-cholestenol-(6β)-on-(3) [1]	27	±
i-cholesterol methyl ether [2]	28	—
Cholesteryl chloride [2]	27	—
Δ^2-cholestene [2]	27	—
$\Delta^{1,4}$-cholestadienone-3 [1]	27	—
3β-chlorocholestane [1]	27	—
Δ^5-cholestene [2]	28	—
Cholesterine palmitate [2]	43	—
Ergosterol [1]	28	+
Ergosterone [1]	28	+
Ergost-7-enol [2]	28	+
Ergost-7-enol acetate [2]	30	—
Stigmasterol [2]	29	+
Stigmastadiene-one [1]	29	+
Sitosterol [1]	29	+
Diosgenin [1]	28	+

[a] Cholesterol-filipin concentrations were 4/1.
[b] Name of the compound used is the designation given by the supplier.
[c] Number in brackets indicates solvent: 1 = ethanol (95%); 2 = acetone, 3 = chloroform.
[d] Growth measured turbidimetrically.

1) Oxidation of the 3-β OH to a ketone does not cause loss of activity; more drastic oxidation to a carboxyl greatly reduces it, whereas substitution by a chlorine group destroys the antifilipin effect.

2) Reduction of the Δ^5 double bond with the retention of the hydroxyl or ketone group in the 3-β position does not vitiate biological activity. In the presence of the ketone group, a shift of the double bond to the Δ^4 also causes no change. A more drastic change, however, such as an additional bond as in $\Delta^{1,4}$ cholestadienone, destroyed the effect.

3) Even in the absence of a hydroxyl group or ketone in the 3 position, a Δ^3 double bond activates the molecule as in $\Delta^{3,5}$ cholestadiene. A lone double bond in the Δ^2 or Δ^5 position in cholestene does not have this effect.

4) The ability of cholesterol esters to prevent antibiotic activity varies. The acetate ester of cholesterol was very effective, whereas the palmitate had no activity. This difference is not simply due the length of the acid chain, for the acetate ester of another active compound, ergost-7-enol, was inactive.

Ergosterol and stigmasterol can also be somewhat altered and retain antifilipin activity; ergosterone, ergost-7-enol, and stigmastadiene-one were active. The importance of carbon 3 in the ring is evident in that specific substitutions on the 3 position, even its unsaturation, allows the antifilipin characteristic to be expressed if the long chain is present. The one exception, the C_{19} compound androstanol-(3β)-one-17, which has a hydroxyl in the 3 position but no long side chain, is puzzling. The activity of this compound does serve to emphasize another important aspect of structure, the stereochemical configuration of the molecule; androsterone, which has an α-hydroxyl group on carbon 3, is inactive, whereas the stereosomer, with a β-hydroxyl, antagonizes filipin.

Concentration responses.—Filipin inhibits fungi at low antibiotic levels. *S. cerevisiae* is inhibited for at least 16 hours on the rotary shaker by an initial concentration of 2.0 μg/ml. The active concentrations are probably lower since the antibiotic is gradually inactivated under these highly aerobic conditions. The growth response to a linear series of concentrations of filipin from 0.1 to 2.0 μg/ml does not have a straight-line relationship over the entire range of inhibitory concentrations (Fig. 5), which is probably due to the complicating factor of inactivation. The inhibitory response gives a very steep slope.

Filipin does not inhibit the lag phase of growth more than the log phase. The general effect of filipin, added to liquid media at the time of inoculation with *S. cerevisiae* in concentrations below those that completely prevent growth, is to increase the lag phase. Once the lag phase begins, the slope of growth with time is similar to that of the controls containing no filipin (Fig. 6). Exceptions occur when the levels

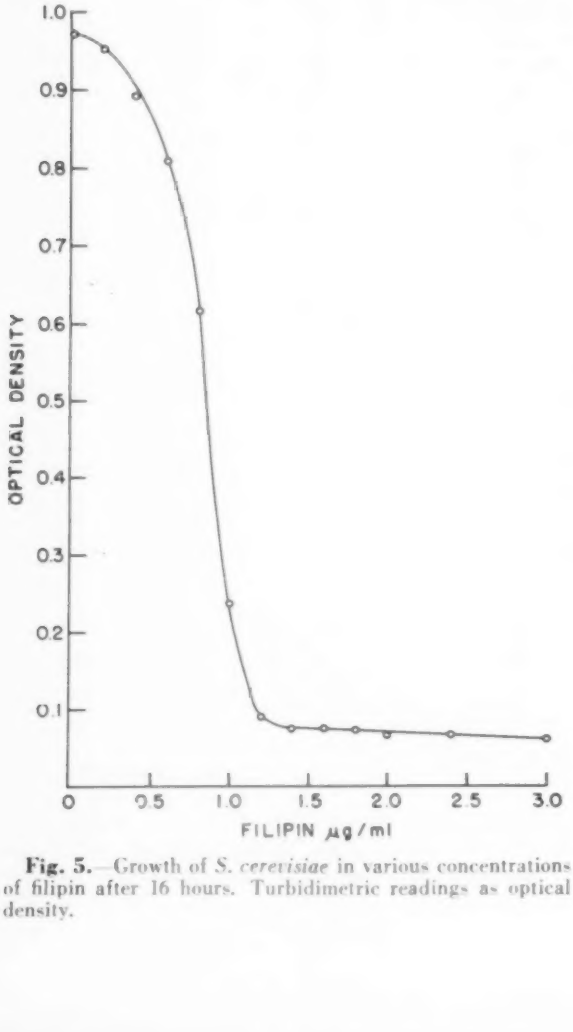

Fig. 5.—Growth of *S. cerevisiae* in various concentrations of filipin after 16 hours. Turbidimetric readings as optical density.

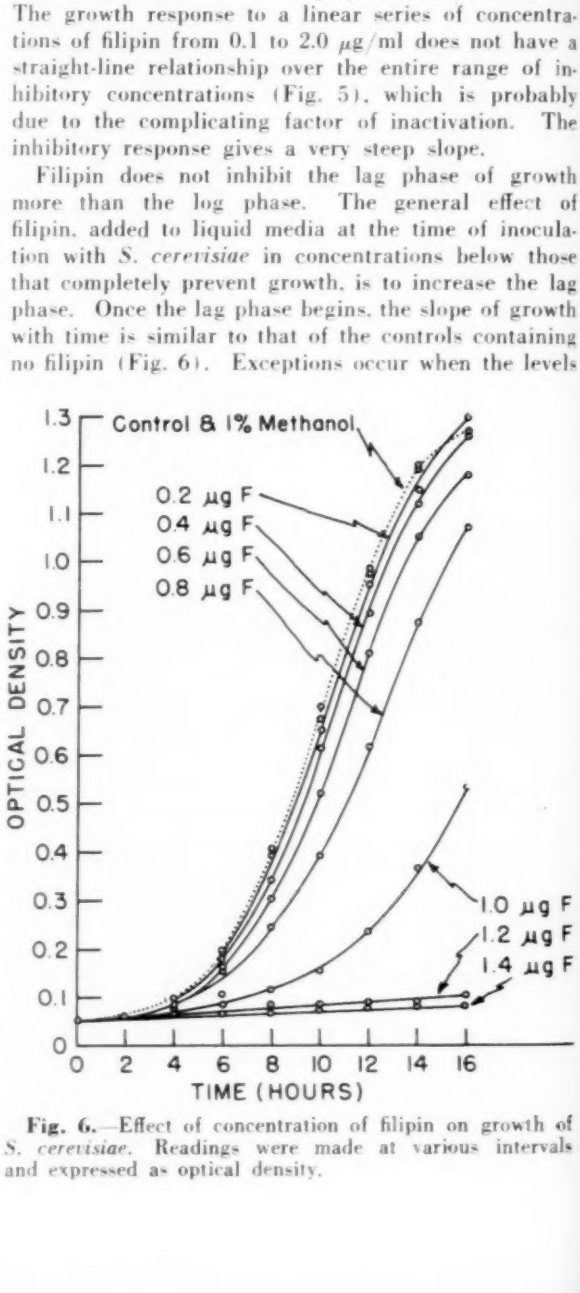

Fig. 6.—Effect of concentration of filipin on growth of *S. cerevisiae*. Readings were made at various intervals and expressed as optical density.

Table 3.—Sterol content of fungi and concentration of filipin needed to prevent growth.

Organism	Inhibitory filipin conc.* (μg/ml)	Ergosterol (% dry wt.)	Total sterol (% dry wt.)
Aspergillus flavus (#58)	20	.001	.01
A. niger (#60)	5	.16	.46
Aspergillus sp. (73)	20	.007	.02
Fusarium lycopersici	10	.23	.68
F. oxysporum	10	.03	.08
H. subpelliculosa	10	.34	.88
P. oxalicum	10	.1	.28
Saccharomyces cerevisiae (F-1)	5–10	.29	.84
S. cerevisiae (Y-132)	5	.28	.94
S. globosus (Y-43)	5	.15	.40
S. pastorianus (Y-46)	10	.75	2.32
Zygorhynchus moelleri	20	.11	.53

* In agar dilution plates.

of antibiotic used are near those providing complete inhibition of growth.

The antagonism of cholesterol to filipin is graphically illustrated in Fig. 7. Cholesterol has no effect on the growth of *S. cerevisiae* in the absence of the antibiotic. At 3 concentrations of filipin—5, 10, and 20 μg/ml—there is increased growth as the sterol is increased by identical steps. The antagonism between these two compounds appears not to be due to a simple competitive inhibition, since the same ratios of filipin to cholesterol at different concentrations of the antibiotic do not allow equal growth of *S. cerevisiae*.

Sterol content of fungi and filipin sensitivity.—Since sterols protect fungi of different sensitivities from inhibition by filipin, the response of an organism to the antibiotic might be governed by its sterol content. Yeasts and filamentous fungi grown in YNBD medium for 5 days on a reciprocal shaker were harvested by either centrifugation or filtration, washed twice with distilled water, dried, and analyzed for ergosterol and total sterol. Data in Table 3 summarize the sterol content of these organisms. Ergosterol varied from 0.001 to 0.34% of the dry weight, and total sterols varied from 0.1 to 2.32% of the dry weight, whereas the sensitivities varied from 5 to 20 μg/ml filipin for complete inhibition. In another experiment, in which the sterols of yeast alone were determined and in which 5 strains of *S. cerevisiae* were included, the total sterols on a wet basis varied from 0.09 to 0.64%. Results from both experiments showed no correlation between the sensitivity of the organism to filipin and its sterol content. Another approach to this problem was made by growing *S. cerevisiae* in YNBD containing levels of cholesterol of 0, 5, 10, 20, and 40 μg/ml, then washing the cells 3 times with water, and inoculating media containing various levels of the antibiotic with these yeasts. Growth in the cholesterol solutions produced no resistance to filipin. A similar experiment with *S. globosus* resulted in increased filipin resistance, but this increase was not related to the sterol concentrations on which the organisms had been grown.

Inhibition of respiration.—Filipin hinders both aerobic and anaerobic respiration of whole cells of *S. cerevisiae* with increased reduction of oxygen uptake or carbon dioxide production as the concentration of the antibiotic increases. In both aerobic and anaerobic respiration, antibiotic levels of 20 μg/ml were sufficient to stop the respiration of about 1.5 mg dry wt. of cells. The ratio of filipin to cells that is required to stop respiration is much greater than needed

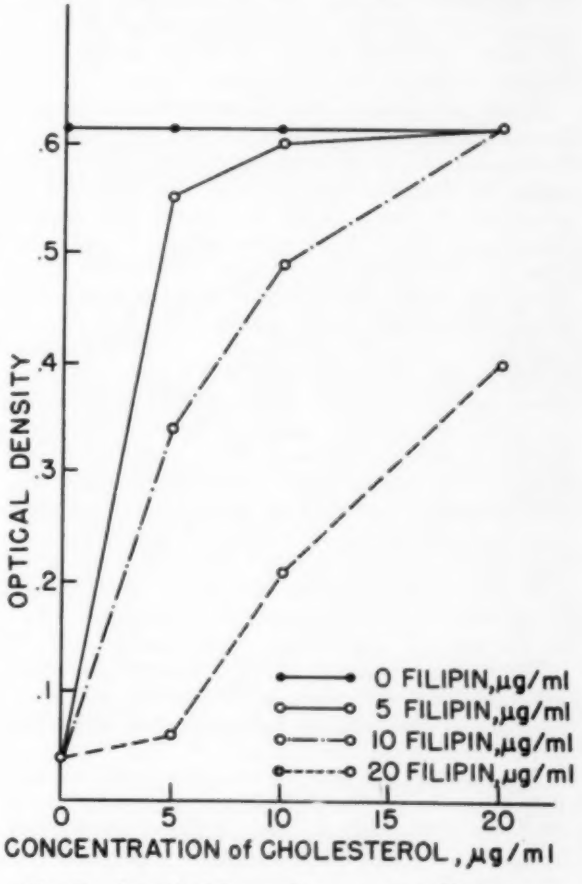

Fig. 7.—Growth of *S. cerevisiae* at various filipin concentrations when different concentrations of cholesterol are added. Growth expressed as optical density.

to prevent growth—respectively 0.144 and 0.027 μg per μg yeast.

Filipin-cell ratio required for inhibition.—The ratio of filipin to cells required for inhibition of *S. cerevisiae* is not constant, and varies with the concentration of cells. Three ratios of filipin to cells (w/w) were used: 0.018:1, 0.029:1, and 0.036:1. Dry wt. of cells were 0.056, 0.168, 0.280, 0.560, and 0.840 mg. No inhibition occurred at any filipin-cell ratio with the lowest cell concentration. The second cell concentration was inhibited only 32%, even at the highest ratio. The remaining three cell concentrations had equal amounts of inhibition at the 2 highest ratios, respectively about 9 and 100%. At the lowest ratio of filipin to cells, however, inhibition was as follows: 0.280, 0.560, and 0.840 mg cells were respectively inhibited at 0, 50, and 80% growth. Thus, for suspensions containing greater total amounts of yeast, the relative amounts of filipin required for inhibition of growth are lower.

Acquisition of resistance.—Neither *S. cerevisiae* nor *H. subpelliculosa* readily acquires resistance to filipin. Cells of *H. subpelliculosa* that were inhibited from growing by the antibiotic for 120 hours in shake tubes and that later grew, were no more resistant to the antibiotic than the original inoculum. Repeated attempts to grow a resistant population of the *S. cerevisiae* by serial transfer in steps of 1.0 μg/ml failed to produce a filipin-resistant yeast.

Action of cysteine and glutathione.—A personal communication has come to our attention in which the investigators report that filipin prevents spore germination of *Rhizopus nigricans* and that this effect is prevented by the addition of cysteine and reduced glutathione (16). These observations brought into question the singularity of sterol effects and a possible relationship between both types of antagonism. Using the gradient plate method with filipin in the top layer and either cysteine or reduced glutathione in the bottom, the phenomenon was studied with three organisms, *S. cerevisiae*, *Zyghorynchus molleri*, and *A. flavus*. Our results confirmed these findings, that the two reducing substances also prevent the growth inhibition by filipin. The concentrations of cysteine and glutathione required to prevent inhibition, however, were of a different order of magnitude than concentrations of the sterols. With filipin at 0.0175 m*M*, the inhibition could be vitiated by 0.0065m*M* of cholesterol, but required 3m*M* of cysteine or 4.5m*M* of the reduced gluthathione. The sterols were 450 to 700 times as active as the reducing compounds, indicating that the mechanisms involved in the antagonism of the reducing substances and that of the sterols were probably different.

Discussion.—Perhaps the most important results from these researches is a concept of the limits of the filipin inhibition and the antagonism by sterols. Obviously, the action of the antibiotic involves some mechanism that is general to fungus physiology or even morphology since filipin inhibits more than 60 species of fungi, including 46 genera and many strains of the same species representing every class of the Eumycetes. This mechanism must be different in some way from that found in Eubacteriales, for none of the tested representatives of that order are inhibited by filipin. The antagonism of some sterols or steroids to the action of filipin involves specific molecular configurations that are attached to the phenanthrene nucleus. Except for the common correlation of the long side chain of 27 or more carbons with activity, none of the other constituents of the molecule generally seem to relate to activity. The wide occurrence of many long-chain sterols in plants and animals —such as sitosterol in plants, ergosterol in fungi, and cholesterol in animals—might furnish a clue that the site of action for filipin could involve the cellular metabolism or function of sterols. The insensitivity of bacteria to this antibiotic might then be correlated with the usual absence of sterols in these microorganisms. Androstanol-3(β)-one-17 is the one exception, for it is a steroid with only 19 carbons, indicating that in some cases the long side chain is not necessary. The action of the sterols is not general to polyenic antibiotics, and might be specific to some molecular structure peculiar to filipin and fungichromin, both of which are pentaenes.

A study of the mechanism of action of filipin was not the prime purpose of the investigation, but the morphological studies could indicate that the cell surfaces are involved (1). The swellings in the wall of *A. flavus* and subsequent bursting, the formation of mycelial filaments consisting of strings of cells by *S. cerevisiae* under growing conditions, and the clumping of yeasts could point to cell surface action. The absence of swelling in *S. cerevisiae* is not surprising, since the yeast cell wall is notoriously tough and very difficult to break. A similar supposition could explain the absence of swelling in some of the other fungi. Preliminary experiments indicate a leakage of some cell components in filipin-treated yeast cells, which also indicates damage to the cell surface.

Data presented herein do not lead to any sound hypothesis for the antagonistic action of some of the sterols and steroids toward the inhibition of fungi by filipin. If there were a direct correlation of ergosterol content or total sterols to the resistance of fungi to filipin, one would have more support for a vital relationship in this phenomenon. Nor does the fact that yeast cells grown in cholesterol have no more resistance to the antibiotic than does the parent strain help in any facile explanation of this antagonism. Further studies on the mechanism of inhibition by filipin and the sterol relationships are under way in hope of elucidating the mode of action of this antibiotic.

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THE INHERITANCE OF X-RAY-INDUCED MUTATIONS TO VIRULENCE IN A UREDIOSPORE CULTURE OF RACE 1 OF MELAMPSORA LINI

H. H. Flor

Plant Pathologist, CRD, ARDS, U.S.D.A., Fargo, North Dakota.

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SUMMARY

Two X-ray-induced mutations to virulence to the flax variety Koto of a culture of race 1 were selfed. Race 1 was heterozygous for avirulence to Koto. The mutations were heritable, as each of the 198 selfed cultures was virulent to Koto. Sixteen of the selfed cultures were also virulent to Abyssinian and Leona, varieties to which race 1 was homozygous for avirulence and whose genes for pathogenicity are closely linked with those for pathogenicity to Koto. Therefore, new genomes for virulence were induced in a rust culture by X-irradiation. The mutations are attributed to the deletion of a portion of the chromosome carrying the closely linked genes for avirulence to Koto, Abyssinian, and Leona. Virulence appears to be conditioned by the absence of the dominant avirulence gene.

The high frequency of radiation-induced mutations to virulence in heterozygous loci of urediospores of a culture of race 1 of *Melampsora lini* (Ehrenb.) Lév. suggests that mutations induced by ionizing radiation are primarily deletions (5, 6). Genetic evidence that avirulence is dominant and that virulence is recessive in the inheritance of the loci involved supports this interpretation (2, 3). If mutations to virulence are the result of chromosomal deletions, then avirulence may be conditioned by the dominant allele and virulence by its absence.

Inheritance of pathogenicity to Koto, Akmolinsk, Abyssinian, and Leona, the differential flax varieties having resistance genes in *P* locus, has been explained by assuming that separate, closely linked genes condition pathogenicity to each (3). Although hundreds of North American rust cultures that attack either Koto or Akmolinsk have been collected, none has attacked both varieties. Consequently, the genes for pathogenicity to Koto and Akmolinsk in North American races are considered to be closely linked and in repulsion. A selfing and test cross study of the culture of race 1 used in the induced mutation experiments showed that it was heterozygous for avirulence to Dakota, Victory A, Koto, and Akmolinsk, and that genes for avirulence to Koto and Akmolinsk were in different nuclei, as would be expected of linkage in the repulsion phase of a heterocaryon (4). The culture of race 1 was homozygous for avirulence to Abyssinian and Leona. Accordingly, its genotype for pathogenicity to

Table 1.—Segregation for pathogenicity of selfed cultures of two race 1 mutations for virulence to Koto.

Pathogenicity[a] of selfed culture to[b]				Number of selfed cultures having indicated pathogenicity	
Dakota	Victory A	Akmolinsk Abyssinian Leona	Koto	Mutant no. 11	Mutant no. 12
A	A	A	V	83	24
V	A	A	V	29	7
A	V	A	V	21	6
A	A	V	V	6	2
V	V	A	V	10	2
V	A	V	V	1	2
A	V	V	V	4	0
V	V	V	V	1	0

$\chi^2 = 26.52^c$, $P < 0.01$
$\chi^2 = 4.14^d$, P lies between 0.70 and .80

[a] A = avirulent; V = virulent.
[b] Race 1, the mutants for virulence to Koto, and all the selfed cultures were avirulent to Ottawa 770B, Bombay, Stewart, Cass, Clay, Polk, Birio, Kenya, Wilden, B. Golden sel., Barnes, Towner, Marshall, Cortland, and Pale Blue Crimped, and virulent to Bison, Williston Brown, Burke, and Ward.
[c] Assuming that avirulence is dominant and independently inherited and conditioned by single genes to Dakota and Victory A and by single closely linked genes to Akmolinsk Abyssinian, and Leona (27:9:9:9:3:3:3:1).
[d] Assuming that single, independently inherited, dominant genes condition avirulence to Dakota and Victory A and that duplicate, independently inherited, dominant genes condition avirulence to Akmolinsk, Abyssinian, and Leona (135:45:45:9:15:3:3:1).

the differential varieties possessing the *P* resistance genes would be $Apap^1Ap^2Ap^3/apAp^1Ap^2Ap^3$ (3).

If the complementary gene hypothesis (3) is true, the ionizing radiation that induced virulence to Koto in a race 1 urediospore deleted or changed the dominant avirulence gene *Ap* to the recessive or its equivalent in the nucleus already carrying the gene for virulence to Akmolinsk. Virulence to Koto and Akmolinsk would then be in the coupling phase in this nucleus. Selfing a mutant virulent to Koto should give cultures attacking both Koto and Akmolinsk, a character possessed by no known North American race. That would establish the heritability of radiation-induced mutations, the need for which was recognized in previous reports (5, 6). Furthermore, the occurrence in the selfed populations of cultures virulent to Abyssinian and Leona in addition to Koto and Akmolinsk, would indicate that virulence resulted from a deletion of a segment of the chromosome rather than from a modification of the dominant alleles to the recessives.

Material and methods.—Two cultures of race 1 mutants virulent to Koto were obtained from Dr. E. A. Schwinghamer. One of these, Culture No. 11, was single-spored before telia of it were produced on Koto; the other, Culture No. 12, was not. Teliospores were developed in the greenhouse during the winter and induced to germinate a few weeks after maturity by alternate wetting and drying on soil under a greenhouse bench. Selfing methods have been described (1). The pathogenicity of each selfed culture to the 25 differential varieties monogenic for resistance was determined.

Experimental results and discussion.—The pathogenicity of 198 selfed cultures of two race 1 mutations for virulence to Koto and the frequency of each variant are shown by data in Table 1. All cultures were virulent to Koto, showing that these mutations caused by X rays were heritable.

Genes that were inherited independently of the gene for pathogenicity to Koto were not altered by the X-ray treatment. This was to be expected, since multiple mutations in separate pathogenicity loci were not likely to occur in a study of only 2 individuals, even at the relatively high mutation frequencies reported for the urediospores of *M. lini* (6). Except on varieties having resistance genes in *P* locus, the pathogenicity pattern of the selfed cultures of the mutants was similar to that of the selfed cultures of race 1 from which the mutants were derived. Each selfed culture was virulent to the varieties to which race 1 was virulent, and avirulent to the varieties to which race 1 was homozygous for avirulence. The 2 selfed populations segregated for pathogenicity to Dakota and Victory A, varieties to which race 1 was heterozygous, into ratios closely fitting those expected if the avirulence of each mutant culture to these varieties was conditioned by a single, independently inherited, dominant gene.

Although race 1 was homozygous for avirulence to both Abyssinian and Leona, the selfed cultures of the mutants that were virulent to Akmolinsk were also virulent to Abyssinian and Leona. This would be expected if virulence is a deletion and the ionizing radiation that induced virulence to Koto in a urediospore of race 1 deleted the segment of the chromosome in which lay the closely linked genes *Ap*, Ap^2, and Ap^3, which respectively condition avirulence to Koto, Abyssinian, and Leona. Leona had been highly resistant to all North American races of the flax rust fungus. Therefore, a new genome highly virulent to a heretofore resistant variety was induced in a rust culture by X-ray treatment.

In hybrids between North American and South American races of *M. lini*, virulence of the latter to Koto, Akmolinsk, Abyssinian, and Leona was recessive and inherited as a unit in a monofactorial ratio. Virulence to these varieties was inherited as a unit in the selfed population of both mutant cultures, but the number of virulent segregates was deficient. The selfed population of Mutant No. 12 was too small for a statistical analysis involving 3 or 4 factors, but the observed ratios of segregates of both mutants did not deviate significantly from those expected if virulence to the varieties having resistance genes in *P* locus was recessive and conditioned by 2 pairs of duplicate genes. However, both nuclei in cultures virulent to the *P* resistance alleles carried the chromosome deletion; whereas neither or only 1 of the nuclei in the avirulent cultures carried it. The doubling of the deleted chro-

mosomes may have had lethal or other deleterious effects, thus reducing the number of virulent cultures.

In host-parasite relationships of the rusts, resistance usually results when the reciprocal or complementary genes in both host and parasite are dominant. Susceptibility usually results when either or both are recessive. These facts suggest that the dominant members of a pair govern the production of substances that are mutually antagonistic or act as antimetabolites. The evidence that virulence is the result of the deletion of the dominant avirulence genes supports this hypothesis and indicates that it is some inhibitive function of the dominant resistance-avirulence genes that characterizes physiologic specialization in the flax rust fungus. This may be an important consideration in studies to determine the physiology of resistance and susceptibility.—State College Station, Fargo, North Dakota.

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INOCULUM PRODUCTION AND SEEDLING EVALUATION OF CELERY FOR RESISTANCE TO CERCOSPORA APII

H. H. Murakishi, S. Honma, and R. Knutson

Respectively, Associate Professor of Plant Pathology, Associate Professor of Horticulture, and Graduate Assistant in Botany and Plant Pathology. Resistant lines of celery were kindly furnished by Dr. H. M. Munger, Dept. of Vegetable Crops, Cornell University. Thanks are also due Richard Crum and Otto Heeckt for technical assistance.

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SUMMARY

Abundant conidia of *Cercospora apii* were obtained on celery leaf-muck soil medium, carrot leaf decoction agar, and carrot root discs. Respective average number of conidia produced per mm² of media surface were 80, 53, and 42. Carrot leaf decoction agar was preferred because 2–3 successive crops of conidia could be harvested. When a spore suspension containing 5000–10000 conidia per cc was atomized on susceptible seedlings at the 4–6-leaf stage and the plants incubated 48–72 hours at 100% RH, lesions suitable for disease evaluation resulted after 10–14 days in the greenhouse. The reaction of resistant and susceptible lines and varieties to seedling infection were similar to results with natural infection in the field.

Previously, tests to determine the resistance of celery (*Apium graveolens* L.) to the early blight fungus (*Cercospora apii* Fres.) have depended on natural infection in the field (5, 8, 9) because the fungus did not sporulate readily on standard laboratory media (2). Although a successful field test was conducted at East Lansing in 1956, the following year mixed infections with Septoria late blight and aster yellows virus made evaluation extremely difficult. Other problems of field testing of celery have been escapes (5) and unfavorable weather conditions for early blight development (7, 10). Solving the problem required a method of producing abundant spores in culture. Lewis (3) induced sporulation of *C. apii* in a celery leaf-muck soil medium, and Kilpatrick and Johnson (1) found carrot leaf decoction agar an excellent medium for conidium production of 11 other species of *Cercospora*. Miller (4) reported that a diverse group of fungi sporulated better on a vegetable-juice agar medium than on potato-dextrose agar. The following studies were made to test the suitability of these and other media for producing spore inoculum of *C. apii* and to develop methods of screening celery seedlings for resistance to this fungus. Preliminary results have been published (6).

Materials and methods.—*Spore production.*—Each medium was evaluated as to efficiency of conidial production by determining the number of spores produced per unit surface area of the medium. Thus, in comparing agar media, a known volume (50 cc) of warm medium was poured into 250-cc Erlenmeyer flasks to give a surface area of the solidified agar of about 5,000 mm². Plant decoction media were prepared according to the method of Kilpatrick and Johnson (1). Celery leaf-muck soil medium and V-8 juice agar were respectively prepared as described by Lewis (3) and Miller (4). Other media were obtained from Difco Laboratories, Inc., Detroit, Michigan. For media containing no agar, such as carrot discs and celery leaf-muck soil, the combined surface areas of the plant parts used in each case were adjusted so they closely approximated that of the agar media. To each of the 10 media tested were added 2-cc portions of an aqueous spore suspension (5000 conidia per cc). The flasks were gently rotated to ensure as uniform a distribution of spores as possible, and then incubated at 20–24°C. After 9 days' growth, 10 cc of sterile water were added to each of the flasks, which were then placed on a mechanical shaker for 10 minutes. Spore counts were made from three .005-cc samples of conidial suspension from each of the 5 replicate flasks. For most media the trial was repeated, in which case the spore count represented 30 determinations of 10 flasks.

Table 1.—Reaction of celery to natural infection by *C. apii* in the field and to artificial inoculation in the greenhouse.

Line or variety	Disease severity rating[a]	
	Field test, 1956[b]	Greenhouse test[c]
56-823	1.0	1.0
56-921	1.7	0.8
56-824	2.0	2.0
Emerson Pascal	3.0	2.0
Summer Pascal	4.3	5.0
Utah Pascal	5.0	5.0

[a] From 0, very resistant, to 5, very susceptible.
[b] Average of three 20-plant replicates in which plants were naturally infected by *C. apii*.
[c] Average of two 48-plant trials. Plants were artificially inoculated in the greenhouse.

Inoculation technique.—Celery seeds were germinated in vermiculite and transplanted to steamed soil in flats or pots. The susceptible variety Utah 15-B was used in studying inoculation methods. In testing breeding lines and varieties, plants were inoculated twice to minimize the possibility of escapes. The second inoculation was made about 3 weeks after the first. A spore suspension (5000–10000 conidia per cc) was applied with a De Vilbiss No. 261 atomizer at 15–20 lb. air pressure on young seedlings at the 4–6-leaf stage. Inoculated plants were immediately subjected to 100% relative humidity (RH) at 23–25°C in a polyethylene-enclosed chamber equipped with an automatic humidifier and temperature controls. After 48–72 hours, the plants were removed to the greenhouse at 19–30°C, averaging about 23°C. Lesion counts were made 10–14 days later.

Results.—*Sporulation of C. apii.*—The comparative yield of conidia expressed as number of spores produced per mm^2 on each of the 10 different media were: celery leaf-muck soil, 80; carrot leaf decoction agar, 53; carrot root discs, 42; carrot leaf decoction agar, 26; V-8 juice agar, 21; Sabouraud dextrose agar, 13; corn-meal agar, 8; celery-leaf-decoction agar, 5, and potato-dextrose agar, 1. The best media for sporulation were celery leaf-muck soil, carrot-leaf-decoction agar, and carrot discs. For inoculum purposes, however, the carrot-leaf-decoction medium proved most satisfactory since 2–3 successive crops of conidia could be harvested. The plant-tissue media tended to disintegrate after the first harvest.

Effect of age of plant on symptom development.—Plants ranging in age from young seedlings with first true leaves just expanding to year-old plants in 6-in. pots were inoculated with a conidial suspension (1000 spores per cc). The youngest seedlings showed no symptoms for 1 month, whereas all those at the 2–4-leaf stage and older developed early blight lesions in 10–14 days.

Effect of spore concentration.—Spore concentrations of 500, 1000, and 10,000 conidia per cc were tested on 3 groups of seedlings at the 4–6-leaf stage. On plants atomized with a concentration of 500 spores per cc, an average of 2–3 lesions developed per leaf. With 1000 spores per cc, 4–9 lesions per leaf resulted, and 10,000 spores per cc produced 5–15 lesions per leaf.

Effect of length of incubation at 100% RH.—Seedlings were inoculated with a spore suspension containing 5000 conidia per cc and placed in the humidity chamber at 100% RH. At intervals of 1–10 days, groups of plants were removed from the chamber and placed on a greenhouse bench. The results were: no lesions after only 24 hours in 100% RH; 3–6 lesions per leaf after 48 hours; 4–8 lesions after 72 hours; 5–30 after 5 days; 8–40 after 7 days; and 6–50 after 10 days. Based on suitability for lesion counting, the optimum incubation period in the humidity chamber was 48–72 hours. More lesions developed after 5 days or longer, but they often became confluent and contributed to a rapid collapse of the tissue.

Comparison of seedling-infection method with field test.—In 1956, 25 breeding lines and varieties were evaluated in the field for early blight resistance based on a disease severity scale modified from Townsend et al (9)—from 0 for very resistant, with no lesions, to 5 for very susceptible. The average of all individual plant ratings in the 3 replicates were then determined for a single line or variety. For the seedling infection method, 6 of the 25 lines and varieties tested in the field were artificially inoculated and evaluated. In 2 greenhouse trials, 96 plants of each of the 6 lines and varieties were evaluated. Since the results of the 2 trials were closely similar, the data were combined. As shown in Table 1, the results of the greenhouse tests are generally in agreement with those obtained in the field.

For the 1959 early blight evaluations, 2930 F_2 seedlings resulting from 12 crosses made to obtain resistance were first screened in the greenhouse following 2 successive inoculations. Those surviving were 38% of the original population. For further testing of horticultural characters as well as early blight resistance, the survivors were planted in a field plot. The area used for growing these plants was about one-third of that required had not the seedling method of inoculation been used.

Conclusion.—The greenhouse seedling method of inoculation minimizes the possibility of mixed infections as well as escapes, thereby enabling a more precise separation of resistant and susceptible plants in a segregating population. Screening all but resistant plants in the greenhouse reduces the amount of field work to a minimum.—Department of Botany and Plant Pathology, Department of Horticulture, Michigan State University, East Lansing.

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PHYSIOLOGY OF AN ISOLATE OF FUSARIUM OXYSPORUM F. CUBENSE

Eugene M. Wilson

Plant Pathologist, Central Research Laboratories, United Fruit Company, Norwood, Massachusetts.

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SUMMARY

The number of microconidia and chlamydospores produced by an isolate of *Fusarium oxysporum* f. *cubense* varied with the level of carbon and nitrogen and the method of culture. Low levels of carbon, added as sucrose, favored chlamydospore formation of standing or shaken cultures in liquid media. The formation of microconidia was favored by high concentrations of carbon in medium containing agar. Exposure of young mycelia to ultraviolet light increased the number of macroconidia formed. As O_2 concentration increased from 0.01% to 15% in a nitrogen atmosphere, production of microconidia increased. Colonies developed under low O_2 concentration produced macroconidia earlier when returned to a normal atmosphere than did colonies held at relatively higher O_2 concentrations. On rhizome tissue of Gros Michel banana plants fungal growth was abundant at 2.5 or 1% O_2 in a nitrogen atmosphere, and discoloration of the cut surface was less and decay deeper than at higher O_2 concentrations. Microconidia had a greater oxygen uptake than either macroconidia or chlamydospores. Macroconidia and chlamydospores germinated during the first 5 hours in Warburg flasks at 29.7°C, but microconidia produced germ tubes only after 9–14 hours. The greater the number of microconidia per unit volume of medium, the lower was the per cent germination and increase in spore volume. In distilled water these spores germinated only when in low concentrations, whereas macroconidia and chlamydospores germinated at high concentrations. Microconidia appeared to double in size before germination. The fungus synthesized an exocellular oligosaccharide (R_F 0.05) from a synthetic medium containing sucrose as the carbon source. All 3 spore forms survived –62°C. Chlamydospores survived lyophilization from the frozen state (pre-frozen at –62°C); conidia did not.

The physiology of wilt-inciting *Fusaria* has been commonly studied with two objectives: 1) classifying these fungi by physiological variation (1, 3, 5, 6), and 2) relating the physiology of a certain *forma* to the disease phenomena incited by that *forma* (6, 7, 8, 14). The physiology of sporulation, spore-germination, and spore-survival of this group of fungi has received a little attention (2, 11).

Sporulation, spore germination, and spore survival by *Fusaria* in the field are influenced by the many variables in soils. There are no reliable methods for studying these physiological processes as they occur in the soil. As an alternative, arbitrary conditions may be studied.

The present investigations were made to determine the influence of certain nutritional and environmental factors on the sporulation and behavior of spores of an isolate of *Fusarium oxysporum* f. *cubense* (E.F.Sm.) Snyd. & Hans.

Materials and methods.—A monospore isolate of *Fusarium oxysporum* f. *cubense* obtained from Mr. B. H. Waite, University of California, Berkeley, was used. This isolate had been found associated with the yellowing syndrome of the Panama disease of bananas (17). Its pathogenicity was repeatedly demonstrated with small Gros Michel banana plants in the greenhouse. It produced an abundance of microconidia, only sparse macroconidia, and chlamydospores on PDA (potato-dextrose agar). For these studies a monospore culture was maintained by transferring it weekly to fresh PDA. This isolate appeared to be stable when so maintained, although some isolates from aged sporodochial-producing cultures were strikingly different in colony type and growth characteristics.

Microconidia that were borne on 5–6-day-old colonies were suspended in water with the aid of a wire loop. Chlamydospores were obtained from colonies on 30% V-8 juice (Campbell Soup Company, Camden, New Jersey) and 2% agar medium or on PDA, where they formed in abundance within 2 weeks. Large masses of mycelium were stripped-off the medium, and the chlamydospores were separated from their connecting hyphae with a Virtis homogenizer. The conidia and some fragments of mycelium were filtered-off with a cloth filter. Macroconidia were harvested from a variant of this isolate, which produced up to 85% macroconidia on either PDA or the V-8 juice medium. This variant was a pionnotal isolate that

Table 1.—Production of chlamydospores* as affected by concentrations of carbon and nitrogen.

Nitrogen g/L	Carbon g/L 0.0	0.26	0.53	1.05	2.10	4.20	8.42	Nitrogen grand mean
Liquid media								
0.022		9.2	6.3	2.7	1.6	0.8	0.7	3.55
0.044		10.0	7.0	1.8	1.6	0.8	0.1	3.55
0.087		10.0	7.0	5.5	2.8	0.5	0.0	5.16
0.175		9.8	7.8	5.3	2.8	0.5	0.0	5.24
0.349		8.2	5.8	4.3	1.8	0.5	0.0	4.12
Carbon grand mean		9.44	6.78	3.92	2.12	0.62	0.16	
LSD (P = 0.05) carbon grand mean = 2.34								
Solid media								
0.0	1.0	0.5	0.5	0.0	0.5	0.0	0.5	0.43
0.022	1.0	5.5	4.0	6.5	1.0	0.5	0.5	2.71
0.044	0.0	3.5	6.5	6.0	8.0	2.5	0.0	3.78
0.087	1.0	4.0	4.0	6.0	6.5	4.0	0.5	3.71
0.175	0.5	5.5	6.5	8.5	10.0	9.5	9.5	7.14
0.349	0.5	4.5	7.0	9.0	9.5	9.5	10.0	7.14
Carbon grand mean	0.66	3.91	4.75	6.0	5.91	4.33	3.5	
LSD (P = 0.05) nitrogen grand mean = 2.64								

* Chlamydospores per unit of mycelium based on an arbitrary scale of 0–10.

arose from an old culture of the original isolate. The pionnotal isolate was found necessary to get more homogeneous preparations of macroconidia. This fungus was also found to be pathogenic to Gros Michel banana plants. All cultures were maintained at 28°C in an incubator except where otherwise indicated.

Preparations of spores were washed at least 3 times by centrifuging and resuspending them in sterile distilled water. No preparation was completely free of other spore-forms, for the technique of getting preparations of any one spore-form was imperfect, though purity was about 85%.

All treatments were replicated 3 times or more, and the experiments were repeated at least once.

Experiments and results.—*Effects of carbon and nitrogen on production of chlamydospores and conidia.*—Culture vessels were 125-ml Erlenmeyer flasks or 9-cm Petri dishes. The standard volume of medium was 25 ml. A basal medium (basal salts) of 2.22 g $MgSO_4 \cdot 7H_2O$, 3.46 g KH_2PO_4, and 1.105 g K_2HPO_4 in 1.0 L of distilled water was amended with sucrose and NH_4NO_3 to give varying levels of carbon and nitrogen and C/N ratios. Bacto-Agar (2%) was added for the cultures in Petri dishes. All chemicals were Baker Analyzed Reagents. In seeding media in flasks, an aqueous suspension of conidium was added as a drop jarred from a wire loop (5.0-mm diameter). In seeding plates this drop was applied to the surface near the center.

Standing cultures, plate cultures, and cultures that had been shaken on a rotary shaker were observed 3–30 days after seeding for form and number of spores produced. An arbitrary scale of 0–10 was used in estimating the number of a particular spore-form produced per culture. Cultures on liquid media were free of pellets and clumps of mycelium and were examined by placing a standardized loopful of the suspended conidia on a slide and covering them with a uniform coverslip. About equal volumes of suspensions were observed immediately after the slides were prepared. Samples from cultures on solid media were prepared by adding 5 ml of sterile water to the culture, rubbing its surface with a wire loop, and placing a loopful of the resulting suspension on a slide. The numbers of chlamydospores per unit of mycelium were estimated and recorded on the 0–10 arbitrary scale.

In experiments conducted at room temperatures and in room light few macroconidia were produced on these media. Their formation apparently was not correlated with the concentration of carbon and nitrogen or the C/N ratio of the medium. Stimulation of production of macroconidia by light was indicated by comparing these cultures with similar cultures in the dark. A similar effect from ultraviolet light is considered later herein.

In both standing and shaken liquid media the formation of chlamydospores was significantly favored by low levels of carbon, but on media solidified with agar the formation of chlamydospores was favored by high nitrogen levels (Table 1).

The average number of microconidia produced was greater per unit of shaken medium than per unit of standing medium (Table 2). More microconidia was formed at low than at high levels of nitrogen. Few microconidia were formed when either the nitrogen or carbon source was omitted.

The production of microconidia from solid media was favored by high levels of carbon (Table 2). Microconidia formed when carbon and nitrogen were omitted from these media, but more formed when carbon was present.

Variations in these data could not be statistically (P = 0.05) assigned to an effect of carbon and nitrogen interaction.

Influence of ultraviolet light on formation of macroconidia.—About 11×10^9 spores in 20 ml of an aqueous suspension were added to 980 ml of unsolidified PDA. The resulting suspension was divided equally among 40 Petri dishes. After one day of incubation in darkness they were exposed to ultraviolet light for varying

Table 2.—Production of microconidia[a] as affected by concentration of carbon and nitrogen.

Nitrogen g/L	Carbon g/L 0.0	0.26	0.53	1.05	2.10	4.20	8.42	Nitrogen grand mean
Liquid media								
Standing culture								
0.0	0.0	0.0	0.0	0.0	0.5	0.5	0.5	0.08
0.022	0.0	2.3	3.6	1.3	3.0	2.0	1.8	2.33
0.044	0.0	1.8	2.1	2.5	2.1	5.1	5.1	3.11
0.087	0.0	1.6	3.1	3.6	2.3	3.3	2.6	2.75
0.175	0.0	1.2	2.3	0.6	0.5	0.5	2.0	1.25
0.349	0.0	0.8	1.5	0.6	0.3	0.3	0.3	0.63
Carbon grand mean	0.0	1.28	2.10	1.43	1.45	1.95	2.05	
LSD (P = 0.01) nitrogen grand mean = 1.35								
Shake culture								
0.0	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
0.022	0.0	4.0	5.3	5.6	6.3	6.0	5.3	5.42
0.044	0.2	1.6	4.3	4.0	6.6	6.6	6.3	4.90
0.087	0.0	4.6	6.0	5.6	7.0	5.3	2.3	5.13
0.175	0.0	2.3	4.6	5.0	3.3	5.0	4.0	4.03
0.349	0.3	1.5	1.8	4.0	4.0	2.6	2.3	2.7
Carbon grand mean	0.11	2.41	3.75	4.11	4.61	4.33	3.45	
LSD (P = 0.05) nitrogen grand mean = 2.06								
Solid media								
0.0	0.5	2.0	2.5	3.5	3.0	3.0	5.0	2.78
0.022	1.5	5.0	7.0	9.0	9.5	10.0	9.5	7.30
0.044	1.0	4.0	5.5	9.0	9.5	10.0	10.0	7.00
0.087	1.5	3.5	7.0	9.0	9.0	10.0	10.0	7.14
0.175	2.0	4.5	5.5	9.0	10.0	10.0	10.0	7.28
0.349	0.5	4.5	6.5	8.5	9.5	10.0	10.0	7.07
Carbon grand mean	1.16	3.92	5.66	8.0	8.41	8.83	9.08	
LSD (P = 0.10) carbon grand mean = 1.72								

[a] Microconidia per culture based on an arbitrary scale of 0–10.

intervals about 5 ft. from a Hanovia 94A-1 sterilization lamp. These cultures were incubated at room temperatures, and after the treatment with ultraviolet light were incubated in the laboratory.

The production of macroconidia was determined by adding 10 ml of sterile distilled water to the surface of the cultures 3–10 days after exposure, rubbing the surface of the culture, and observing the number of macroconidia contained per microscope field under a uniform coverslip.

An increase in the production of macroconidia occurred after the culture had been exposed to ultraviolet light for 4 minutes. Exposures of over 32 minutes were apparently less effective than lower dosages.

When conidia from old cultures were used for seeding the medium, almost as many macroconidia were formed in the dark as were formed after various exposures to ultraviolet light.

Growth and sporulation in controlled atmosphere.—The influence of different concentrations of O_2, CO_2, and nitrogen on growth and sporulation was determined with PDA-containing Petri dishes placed in desiccators. The nitrogen contained 0.01% oxygen according to the manufacturer's analysis (New England Gas Products, 27 West Street, Charlestown, Massachusetts). The desiccators in which the gases were measured had been evacuated with a water aspirator and infiltrated three times with tank nitrogen. Microconidia on the surface of PDA in these desiccators germinated and formed short hyphae in an atmosphere of 0.01% O_2 in nitrogen. These hyphae produced few spores and had limited growth. As the concentrations of O_2 increased, the number of microconidia increased per unit of mycelium. Colonies that developed under a low concentration of O_2 and were returned to a normal atmosphere produced macroconidia earlier than did colonies exposed to higher concentrations of O_2 (Table 3). Macroconidia were found on both the old and new growth.

Experiments with a Dubnoff metabolic shaking incubator indicated that the low-O_2 effect was related to the substrate in which this fungus was grown. With the basal salts, sucrose, and NH_4NO_3 in a liquid culture medium that favored growth of this isolate in a normal atmosphere, growth was only sparse, whereas 0.2% yeast extract (Difco) added to this medium

Table 3.—Production of conidia as affected by concentration of oxygen in a nitrogen atmosphere.

Oxygen (%)	Growth[a]	Microconidia[b]	Macroconidia[c]
0.01	30	2	10
1.0	33	4	10
2.5	41	6	4
5.0	56	8	2
10.0	60	8	0
15.0	59	10	0
Check	72	10	0

[a] Diameter of growth (mm) in 7 days.
[b] Microconidia per unit of mycelium, based on arbitrary scale of 0–10.
[c] Macroconidia per unit of mycelium, based on arbitrary scale of 0–10, two days after removing from controlled atmosphere.

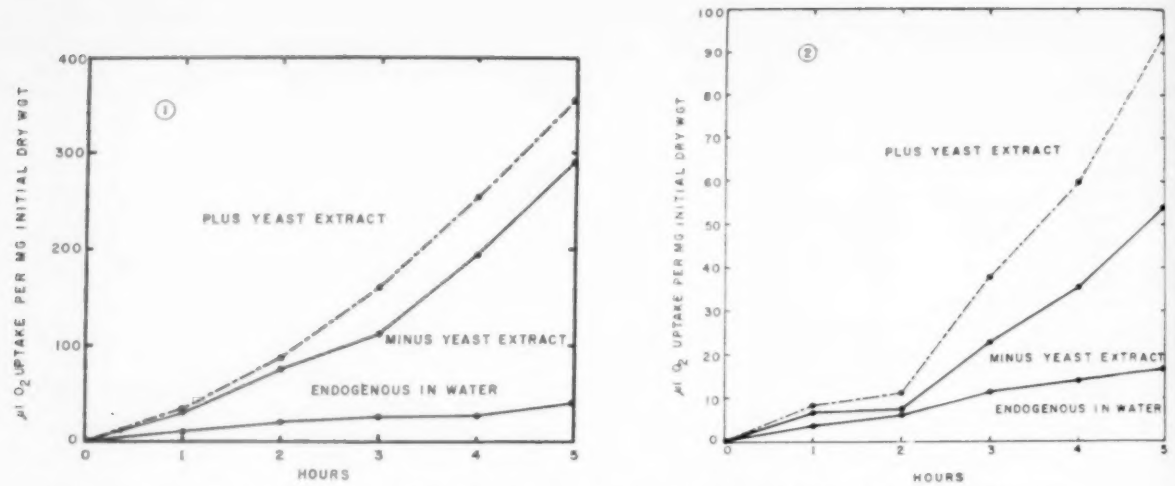


Fig. 1–2.—Respiration of **1)** microconidia and **2)** macroconidia during incubation in a basal medium.

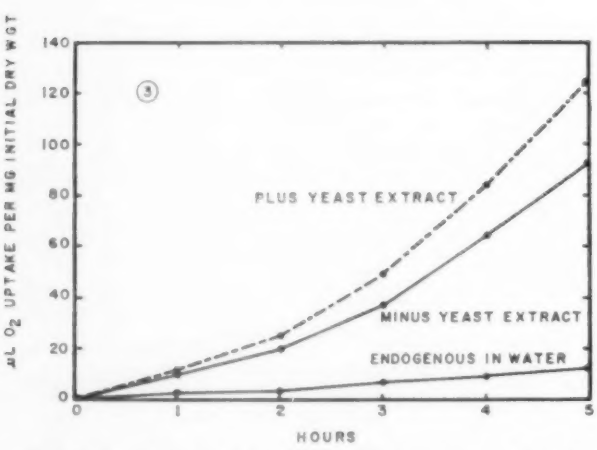
resulted in considerable growth in a nearly pure (0.01% O_2) nitrogen atmosphere. In an atmosphere of CO_2, microconidia germinated and formed germ tubes that produced chlamydospores. Yeast extract did not increase the amount of growth in this medium in an atmosphere of CO_2.

Growth was abundant on rhizome tissue of Gros Michel banana plants incubated in a nitrogen atmosphere at low oxygen levels. Discoloration of the cut surface was less and decay was deeper at O_2 concentrations of about 5, 2.5, or 1% than at higher O_2 concentrations.

Respiration of spores.—Preparations of either microconidia or chlamydospores were placed in a medium containing 1.48 $MgSO_4 \cdot 7H_2O$, 2.32 g KH_2PO_4, 0.74 g K_2HPO_4, 6.66 g sucrose, and 0.83 g NH_4NO_3 in 1 L of distilled water, with or without 0.13% yeast extract. One milliliter of aqueous suspension of spores was added to 2.0 ml of autoclaved medium. Oxygen uptake was measured by conventional Warburg manometric techniques.

Oxygen uptake by either chlamydospores, macroconidia, or microconidia varied with different spore harvests. Representative results are in Fig. 1, 2, 3. Oxygen uptake was greater by microconidia than by either macroconidia or chlamydospores. The magnitude of O_2 uptake by microconidia after 5 hours was directly proportional to the dry weight of the spores (Fig. 4). The influence of pH of the medium upon O_2 uptake by either microconidia or chlamydospores was only slight in the pH range 4.4–6.4. Chlamydospores consumed more oxygen than did macroconidia during these respiration studies. The addition of 0.2% yeast extract to the medium increased the oxygen uptake of all spore forms. In water (endogenous respiration), macroconidia and chlamydospores had lower Q_{O_2} values than microconidia.

Microscopic observations of these spore forms



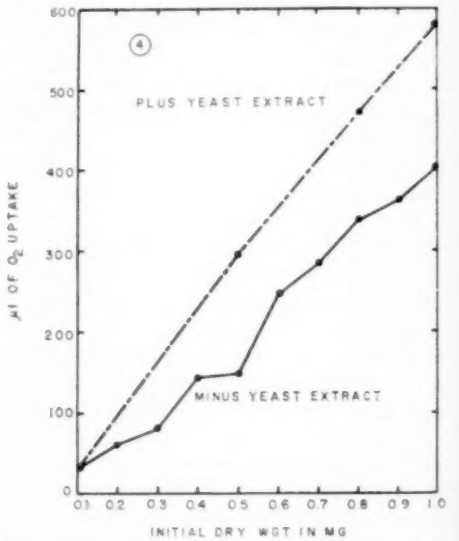


Fig. 3–4.—**3)** Respiration of chlamydospores, and **4)** the relationship between oxygen-uptake and initial dry weight of microconidia incubated for 5 hours in the basal medium.

showed that macroconidia and chlamydospores in a Warburg flask at 29.7°C germinated within the first 5 hours. Microconidia at this temperature produced germ tubes only after 9-14 hours. Evidence that respiration was correlated with a swelling of these spores was a doubling in their size during incubation. With similar media and cultural conditions, the greater the number of microconidia per unit volume of medium the lower was the per cent germination and increase in spore volume. In distilled water these spores germinated only when in low concentrations, whereas macroconidia and chlamydospores germinated in high concentration.

Synthesis of oligosaccharides.—Media containing 20, 10, 5, 2.5, 1.25, 0.625 or 0 g/L of sucrose were seeded with spores. From each flask 0.2 ml of the medium was aseptically removed at each one-day interval during the 10-day incubation period. These samples were analyzed for sugars by paper chromatography according to methods described (19).

Results showed the presence of an oligosaccharide with an R_F of about 0.05, sucrose, and fructose in media containing sucrose above 10 g/L. Glucose was not found in these media.

Sucrose and the oligosaccharide were not present after 6 days of incubation in shake cultures but were found in standing cultures.

Effect of lyophilization on spores.—Spores were suspended in water or fat-free milk and frozen by partly immersing their container, a 115×24-mm centrifuge tube, in a mixture of dry ice and cellosolve. So that the effect of drying under high vacuum could be determined, some suspensions of spores were not prefrozen. The temperature of these suspensions during drying was not determined. About 7 hours were required to bring 5 ml of these suspensions to dryness with a Virtis Roto Freeze at an air pressure of 30–40 μ of mercury.

After either lyophilization or high-vacuum drying, the spore mass was resuspended in water. The suspension was streaked onto the surface of PDA or aqueous agar. Growth after 2 days was used to indicate survival; sparse growth or none indicated that they did not survive.

Microconidia and macroconidia did not survive lyophilization, but chlamydospores did. Microscopic examination of the conidia during incubation showed that the sparse growth that occurred could be explained by traces of chlamydospores that had survived.

Conidia germinated within 24 hours of being frozen in an aqueous suspension at −62°C. High-vacuum drying reduced the per cent germination of conidia as much as 78%.

Discussion.—Increasing the concentration of carbon and nitrogen in soil has been reported to increase the population of *Fusarium oxysporum* f. *cubense* (16). It has not been determined whether this is related to the influence on the sporulation of either the carbon or nitrogen or an interaction of carbon to nitrogen (C/N ratio) or to other factors. During the present study observations with chemically defined media showed that the relative numbers of microconidia and chlamydospores produced by an isolate of this fungus varied with the concentration of carbon and nitrogen of the medium. The C/N ratio in these media appeared to have an influence but this was not statistically significant. Variation also occurred between liquid and solid media and between standing and shaken cultures on a liquid medium. Basal salts in 2% aqueous agar and small quantities of carbon in the form of sucrose supported growth and sporulation of this isolate. The source of nitrogen for this growth was not determined.

Carlile (4) reported that a low C/N ratio favored chlamydospore formation by *F. oxysporum* f. *gladioli* (Massey) Snyd. & Hans. The present results with an isolate of *F. oxysporum* f. *cubense* did not corroborate her report that there was a significant relation between C/N ratio of the medium and formation of chlamydospores. Low concentrations of a carbon source apparently favor chlamydospore formation.

Ramsey and Bailey (12) reported that exposure to ultraviolet radiation favored the formation of macroconidia, and Harter (9, 10) observed an increase in length of the macroconidium as a result of growing *Fusarium* spp. in light. Snyder and Hansen (15) reported an effect of light on taxonomic characters of *Fusarium* spp. Carlile (4) reported that ultraviolet light promoted the formation of macroconidia of *F. oxysporum* f. *gladioli*. The present results with *F. oxysporum* f. *cubense* affirm her observations.

Some isolates of *F. oxysporum* f. *cubense* produce an abundance of pionnotes, covered with macroconidia, whereas other isolates produce only a few macroconidia after incubation for 2 weeks. The isolate used in the present studies had the latter characteristics.

Whether the physiological change resulting from exposures of ultraviolet light is similar to that of aging has not been determined, but the effect of increasing the number of macroconidia that are formed is similar. The possibility of mutation to the pionnotal type from exposures to ultraviolet light may merit further investigation.

An influence of O_2 and CO_2 in the atmosphere surrounding cultures of *Fusaria* associated with wilt diseases has been reported (2, 11, 18). Soil and plant tissue in which these fungi are capable of existing and growing presumably have variable concentrations of these gases. The influence of the composition of the atmosphere surrounding these fungi or the medium on which they exist has not been sufficiently studied.

Observations during the present study indicate that *F. oxysporum* f. *cubense* was capable of growing in low concentrations of O_2 (0.01%). Just as significant was the observation that sporulation occurred at low levels of O_2. The present study on O_2 requirement suggests further investigations to determine if this fungus is a facultative anaerobe. Its growth and sporulation at low concentrations of O_2 may be important from a practical viewpoint.

Thick-walled chlamydospores of fungi are con-

sidered to be dormant, or "resting stage," spores. This has been accepted as being true for chlamydospores of *Fusarium* spp., though there is little evidence to support this conclusion.

Whether the magnitude of O_2 uptake can be assumed to be related to dormancy depends on the criterion of dormancy. This was clearly evident from the present data on respiration of spores. If rates of respiration are accepted as criteria of dormancy, macroconidia and chlamydospores may be considered as dormant spore forms of this fungus. If early formation of germ tubes, i.e., time of germination, however, is the basis for describing dormancy, the microconidium may be considered the more dormant spore form.

Fusarium spp. have been reported to survive lyophilization (13), but survival of the different spore forms of a *Fusarium* has not been reported. Lyophilization apparently destroys conidia, whereas the thick-walled chlamydospores are capable of withstanding the process.

Why high-vacuum drying killed conidia that were pre-frozen in suspension was not determined. The survival of 22% of the conidia exposed to high-vacuum drying without pre-freezing may be explained by the protective nature of the amorphous consistency of the resulting mass of spores.—Central Research Laboratories, United Fruit Company, Norwood, Massachusetts.

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A CRYSTALLINE HATCHING STIMULANT FOR THE GOLDEN NEMATODE

W. V. Hartwell, R. V. Dahlstrom, and A. L. Neal

Respective present addresses of senior authors: Phoenix Field Station Section of U.S.P.H.S., CDA, Phoenix, Arizona; and Research Department, Rahr Malting Co., Manitowoc, Wisconsin.

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SUMMARY

A crystalline preparation that stimulated emergence of golden nematode larvae was obtained from lyophilized leachings. It was extracted from the lyophilized preparation with absolute ethyl alcohol and precipitated in an oil-like state by the addition of diethyl ether. The "oil" was dried and dissolved in a mixture of ethyl and t-butyl alcohols. Adding diethyl ether to this solution precipitated an active crystalline substance. Indications are that the crystalline material contains a hydroxyl group but no free carboxyl group. Analysis of the diphenylphosphate derivative indicated a possible molecular formula of $C_{13}H_{12}O_3$ for the active material.

Triffitt (17) and Hurst (5, 6) were the first investigators to attempt to determine the chemical nature of the natural hatching stimulant (or stimulants) for the golden nematode (*Heterodera rostochiensis* Wr.) larvae. The latter investigator concluded that

the stimulant is an amino acid. A series of investigations by Todd and coworkers (2, 3, 4, 8, 9, 16) led them to conclude that the substance is an acid that possibly contains a lactone group. Other attempts (7, 10, 11, 18) have been made to isolate the active material and study its chemical nature. In each case the final product obtained was a noncrystalline resinous material. A light-amber resinous substance has been obtained in our laboratory by a number of different concentration procedures, and it was finally crystallized by using t-butyl alcohol as one of the solvents.

Materials and methods.—An extension of the procedure of Massey and Neal (11) was used to obtain an active concentrate of the factor. Leachate from soil in which tomato plants (*Lycopersicon esculentum* Mill.) were growing was lyophilized immediately after collection. Twenty grams of the lyophilized leachate was extracted three successive times with 50 ml of absolute ethyl alcohol. The ethanolic extracts were combined and the solvent evaporated off under reduced pressure at room temperature. The resulting residue was extracted with a small volume of absolute ethyl alcohol, and the insoluble material was discarded. About 2 volumes of diethyl ether added to the ethanolic extract caused the formation of a precipitate, which was removed and discarded. More ether was then added to the solution, and any solid material that precipitated was removed after centrifugation. Eventually enough ether was added to cause the precipitation of a light-amber oily material. This "oil" was separated from the supernatant, and formed a light-amber resinous material after drying under vacuum. The resinous material was dissolved in a small volume of a mixture of absolute ethyl and t-butyl alcohols (approx. 1:1 v/v), and diethyl ether was added until a slight turbidity resulted. Microscopic crystals were induced to form by alternately cooling the solution in an ice-salt bath and removing it from the bath for short intervals while small increments of ether were added. The solution was shaken as continuously as possible during this operation. The preparation was placed overnight in a refrigerator at −10°C to permit more nearly complete crystallization. After centrifugation, the supernatant was drained off, and the residue was washed with a small volume of ether and dried under vacuum over P_2O_5. This preparation was labeled A. The weight of the dried sample was 15.5 mg. Three lots of crystalline preparations obtained earlier in this investigation by essentially the same method are labeled B, C, and D. Preparation D was recrystallized 4 times from t-butyl alcohol. About 4 mg was obtained from 1300 L of leachings.

To assay the activity of preparation A, 50 cysts picked at random from a batch of preselected cysts (Belgium, Sand Dune, 1956) were placed in each Petri dish and incubated at 20°C with 20 ml of the solution to be tested. Distilled water was used to make the solutions, and Salts-A (Na, K, Ca, and Mg chlorides) was added as suggested by Robinson and Neal (13, 14, 15). Activity was determined at pH 3. Activity of preparations obtained earlier in this study was determined with Long Island cysts at pH 6–7 without the addition of Salts-A.

The diphenylphosphate derivative of one of the earlier crystalline preparations was made by treating it with diphenylchlorophosphonate prepared according to the method of Brigl and Müller (1). To 18 mg of the crystalline preparation dissolved in 2 ml of ice-cold pyridine was added 20 ml of cold diphenylchlorophosphonate, and the mixture was shaken in an ice bath for 30 minutes, allowed to stand overnight at room temperature, and extracted twice with diethyl ether, 5 ml each time. The ethereal solution was evaporated to dryness under vacuum and the residue dissolved in a minimum amount of absolute ethyl alcohol. The solution was then cooled in an ice bath, and cold water was added dropwise with constant stirring until crystallization was induced. The crystals were separated by filtration (yield, 33 mg) and then recrystallized from absolute ethyl alcohol several times.

Results.—Fig. 1 shows the influence of several crystalline preparations on larval emergence. It was realized that maximum larval emergence would require putting fresh solutions on the cysts each week, but the material was insufficient. Data on the influence of preparation A (Fig. 1) were obtained from 3 replicates on which fresh solutions of the preparation were put on the cysts only after the first week

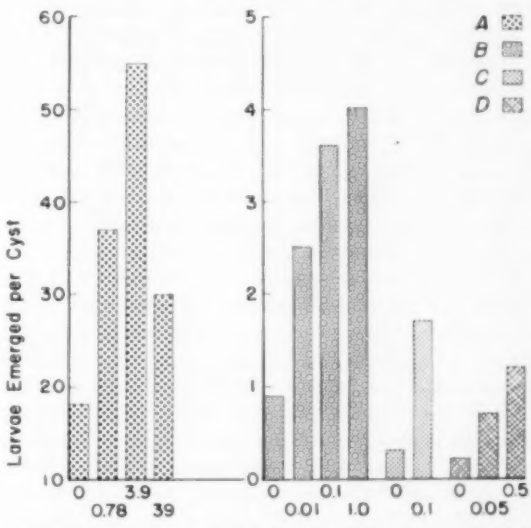

Fig. 1.—Activity of different crystalline preparations as measured by stimulation of emergence of larvae from treated cysts. **A)** assay made on 3 replicates of Belgium, Sand Dune (1956) cysts incubated 2 weeks at pH 3 with Salts-A added. **B, C, D)** assays made on duplicates of Long Island cysts incubated 1 week at pH 6-7 without Salts-A. Preparation D had been recrystallized 4 times from t-butyl alcohol. Statistical significance for mean larval emergence (treatment vs. control) was as follows: **A)** $P > .05$ for 0.78 μg, $P > .01$ for 3.9 μg; **B)** $P > .05$ for all levels; **C)** $P > .10$; **D)** $P > .02$ for all levels.

of incubation and the solutions were aerated once a week during the assay. Preparation A changed larval emergence from that of the water control, increasing it 100% ($P > .05$) at 0.78 μg per ml and 190% ($P > .01$) at 3.9 μg per ml, and inhibiting it at 39 μg per ml, the highest level tested. Larval emergence from the Long Island cysts was very low; nevertheless, the consistent stimulation obtained with 3 different preparations supports the conclusion that the crystalline material is active. In these series of assays, only duplicate determinations were made and the incubation time was one week. Preparation D, which had been recrystallized 4 times from t-butyl alcohol, increased larval emergence 4-fold at a level of 0.5 μg per ml of solution. Mean larval emergence from the cysts treated with the respective preparations was significantly different from that of the water control, as follows: B, $P > .05$; C, $P > .01$; D, $P > .02$.

Infrared spectra of the recrystallized preparation showed the absence of a free carboxyl group and confirmed the conclusion (9) that the active substance contains at least one hydroxyl group. This observation was the determining factor in selecting the diphenylphosphate as a possible derivative. The diphenylphosphate derivative analyzed as follows: C, 66.47, 66.40; H, 4.77, 4.69; O, 19.63; P, 6.85 (average of 4 determinations). (Analysis for C, H, N, and O was made by Huffman Microanalytical Laboratories. Oxygen was determined by the Unterzaucher method, which sometimes fails to give a true value when phosphorus is present.) Only a trace of nitrogen was present (< 0.1%, Dumas; < 0.22%, Kjeldahl), and the melting point was 49° C (uncorrected). The derivative was soluble in ethyl alcohol, pyridine, or glacial acetic acid, and insoluble in water.

Calculations based on phosphorus as the limiting element in the diphenylphosphate derivative indicated a molecular weight of 452 for the derivative and 220 for the factor. The average elemental analysis percentages add up to 97.61, perhaps lowered by interference of phosphorus in the determination of oxygen by the Unterzaucher method. Since another preparation of the diphenylphosphate derivative gave the same elemental analysis within experimental error for carbon and hydrogen, it may be more nearly correct to assume that the value for oxygen should approximate 21.99%. If that value is assumed, the formula for the derivative would be $C_{25}H_{21}O_6P$; molecular weight 448. That would indicate for the factor a tentative formula of $C_{13}H_{12}O_3$ and a molecular weight of 216. Molecular-weight determinations based on the melting point depression of acetic acid were more variable than desired; the average of three determinations on a crystalline preparation, however, was 217 ± 12.

Discussion.—At the time the earlier preparations were obtained, the influence of mineral salts and pH on larval emergence was not recognized. Furthermore, presoaking cysts in vitamin B_6 solution has recently been found (12) to enhance the activity of "deionized" leachings. Unquestionably other substances still unknown must be present in the assay medium for maximum stimulation of emergence. Therefore, one cannot make comparisons of the apparent activity of different preparations assayed under different conditions. For example, the fact that the leachings from which preparation D was obtained gave an average larval emergence of 0.6 larvae per cyst cannot be interpreted to indicate that this preparation was of activity equal to or greater than the starting material. Heavy metals may have decreased the activity of the original leachings, and the absence of certain mineral elements undoubtedly decreased the effect of the crystalline preparation. Also the apparent low larval emergence of 55 larvae per cyst obtained with preparation A may not necessarily indicate that the material is only slightly active. There always remains the possibility that a change in the structure, e.g., racemization, of the active material occurred during its isolation, which might tend to decrease its biological activity without changing its molecular formula. The concentration in the original leachings of the material crystallized is not known since quantitative procedures of isolation have not been attempted. Confirmation of the possible molecular formula of $C_{13}H_{12}O_3$ calculated from the diphenylphosphate derivative and its chemical structure must await further investigations. Further, no conclusion can yet be made on whether this material is the only stimulant present in tomato leachings.—Department of Biochemistry and Nutrition, Cornell University, Ithaca, New York.

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ANTAGONISM AND SYNERGISM AMONG ORGANISMS ASSOCIATED WITH SCALE TIP ROT OF LILIES

J. G. Bald and R. A. Solberg

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SUMMARY

From scale tip rot lesions on Croft lily bulbs a *Pseudomonas* sp. was isolated. In pathogenicity tests it caused lesions on wound-inoculated bulb scales, and was re-isolated from the lesions. *Cylindrocarpon radicicola* and *Fusarium oxysporum* were also isolated from diseased lily bulbs. Alone they produced very slight effects when wound-inoculated on lily scales. Combined with the scale tip rot bacterium and inoculated on lily scales, *C. radicicola* reduced or sometimes eliminated the lesions, whereas *F. oxysporum* grew in the diseased tissue and extended the lesions. These antagonistic and synergistic effects suggest an explanation for anomalous reactions of diseased bulbs to pesticidal treatments.

In some northern California field plots, fungicide-treated Croft lily bulbs (*Lilium longiflorum* Thun.) planted in fumigated soil had little evidence of disease at harvest, except scale tip rot, a condition of unknown etiology (4). Various treatments applied to the bulbs and soil had less effect on incidence of scale tip rot than they had in other trials on symptoms associated with known pathogens (2, 3). Of soil fumigants tested, those containing chloropicrin reduced scale tip rot more than did those containing methyl bromide. There were also unexplained interactions between treatments with soil fumigants and the fungicidal dips, suggesting that the various pesticides might be acting on a complex interacting system of pathogens and antagonists. Attempts were therefore made 1) to find the pathogen causing the disease, and 2) to isolate other organisms capable of influencing its pathogenicity.

McWhorter (4) isolated *Cylindrocarpon radicicola* Wollenw. and *Fusarium* sp. from scale tip rot lesions, but failed to reproduce the disease by inoculating them to Croft lily bulbs. In Holland, van Hell (7) found *C. radicicola* parasitic on the underground parts of lilies, but Ogilvie (5) failed to confirm its pathogenicity. Plakidas (6) inoculated soil with isolates of a *Colletotrichum* from "dieback of the tips of lily bulbs in Oregon" in parallel with *Colletotrichum lilii* Plak., the cause of "black scale" disease, but produced infection in bulbs planted therein only with *C. lilii*.

Materials and methods.—Attempts to isolate the organism causing scale tip rot were made during October, 1958, from bulbs harvested in northern California field plots. Bacteria were found microscopically near the inner edge of active lesions, and the methods of isolation were adapted to reveal a bacterial pathogen. In addition to the plating of tissue fragments, small pieces of tissue were washed 2 days in running water, ground with a pestle and mortar in sterile distilled water, and allowed to stand. Loopfuls of the suspensions were used to streak potato-dextrose agar (PDA) plates or to prepare dilution plates.

The streaks and dilutions yielded several types of bacteria, samples of which were transferred to PDA or beef-peptone agar for further study. One type found to be pathogenic, and two others as checks, were carried through several single-colony isolations to obtain pure cultures. The tissue pieces yielded a mixture of bacteria and fungi.

Bulb scales from pathogen-free Croft lily plants grown in steamed soil were used in pathogenicity tests. Scales were detached from freshly harvested bulbs, and washed in running water before inoculation. A few were sterilized in calcium hypochlorite solution, but most were placed unsterilized in Petri dishes. A suspension of bacteria, generally from a 2-day broth culture, was poured over the scales, half pricked with the sharpened end of a fine wooden applicator stick and half unwounded. Excess inoculum was rinsed out and replaced by a little free water on the bottom of the Petri dish. Inoculated scales were incubated in the dark at temperatures within the range 20–30°C.

Controls were of two kinds, both often present in an experiment: 1) half of a group of scales were unwounded and half wounded in the presence of distilled water under clean but not sterile conditions; 2) half of a group of scales were unwounded and half wounded in the presence of non-pathogenic bacteria also isolated from lily bulbs. One check bulb scale developed a single, not very active, lesion around a puncture; otherwise, on about 200 check scales of both kinds, there were only tissue wound reactions and

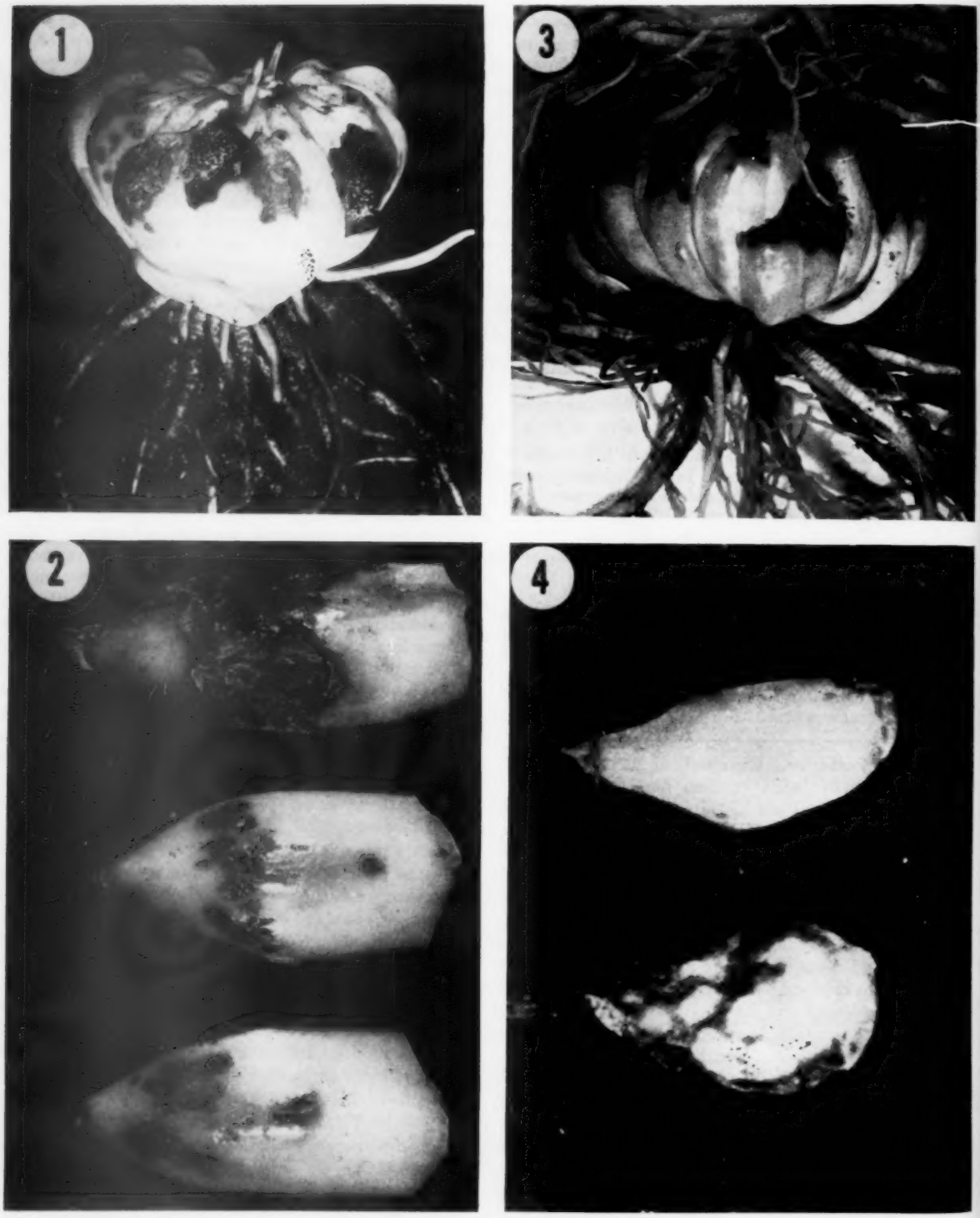

Fig. 1-4.—**1)** Field-grown lily bulb infected with scale tip rot, after storage for 2 months. Rotted scale tips were invaded by fungi, mainly *Fusarium*, which sporulated on the surface. Rhizoglyphid mites were also present in the rotten tissue. Small, round lesions were on other scales; several lesions on the emerging shoot suggested infection by contact with scale tip lesions. **2)** Three scales immediately within the outer whorl. Lesions present were in areas touched or overlaid by scale tip lesions on the outer scales. **3)** Bulb with scale tip rot after forcing in greenhouse. Tissue of lesions rotting or eaten away. **4)** Above, unwounded inoculated scale; below, wound-inoculated with *Pseudomonas* sp. and *Fusarium oxysporum*. The latter is the same scale as in Fig. 5-e. Bacterial rot extended by *F. oxysporum*; sporulating mycelium covers surface.

Table 1.—Particulars of pathogenicity trials with bacteria isolated from scale tip rot lesions and inoculated to pathogen-free bulb scales.

Trial	Description—Type of data	Plate *a* colony, mixed	Plate *b* colonies		
			Single		
			1	2	Mixed
1	Wound-inoculation test—Pathogenicity	+	+	+	+
2	Single-colony re-isolates, from trial 1, wound-inoculated—Number of re-isolates pathogenic	1/3	1/3	2/3	2/3
3	Single-colony subcultures from re-isolates pathogenic in trial 2, wound-inoculated—Sub-cultures pathogenic	3/3	2/3	3/3	2/3
4	Twelve subcultures of trial 3 inoculated on unwounded scales, planted, examined after 5 weeks—Number of scales rotted	2/15	2/15	[a]1/15	1/15
5	Infusions of rotted scales, trial 4, wound-inoculated—Pathogenicity	+	+		+

[a] This bulb scale, though partially rotted, bore a bulbil and root. It was repotted beside the sound scales, but no plant grew.

slight discoloration by saprophytic bacteria that often grew in drops of water collecting over wounds.

Symptoms.—The smaller scale tip rot lesions on blemished and yellow field-grown bulbs are not conspicuous, and only severe symptoms are generally noticed. The tips and perhaps the upper quarter or half of outer scales are rotted, leaving brown, sometimes almost black, remnants of tissue (Fig. 1, 3). If the affected tip has disappeared, the scale tissue along the edge of a lesion appears eaten away, as in part it may be, because the rotten scale tip supports a fauna that includes minute round worms and Rhyzoglyphid mites (4).

On bulbs shipped for forcing there may be confusion between bruising on the tips of outer scales, and the milder symptoms of scale tip rot. If an affected bulb is white and not physically damaged, typical round brown lesions may be seen (Fig. 1). Some may have a slightly sunken center, suggesting that the original point of entry for the pathogen was a small wound (Fig. 2). The lesions are 1–5 mm in diameter or more, lighter brown in color than the disintegrating scale tips, and distributed mainly on the outer surface of the upper halves of the scales. They may be on inner as well as outer scales.

Inoculation trials with the pathogenic bacteria isolated from scale tip rot lesions did not quite reproduce the naturally occurring lesions, but the differences were slight enough to arise from variations of environment. Water-soaked and discolored areas appeared around wounds 24–48 hours after inoculation, darkening to brown. They often became more or less zoned (Fig. 5-g). Under very humid conditions some bacterial ooze appeared on the center. The ooze was surrounded by brown tissue and generally a darker brown rim. Some bacterial isolates induced darker rims around the lesions than did others.

Isolation and pathogenicity tests.—Six PDA plates were chosen from a larger number streaked with suspensions of affected tissues. Three were from scale tip rot lesions, and 3 from tissues with superficial yellowing. Two single colonies and a composite sample of bacteria from each plate were transferred to beef peptone agar, giving 18 bacterial isolates in all. Each was inoculated to 8 pathogen-free lily scales; 4 were wounded by 10–15 stabs, and 4 were not.

None of the isolates from yellowed scales gave lesions. Both single-colony and mixed-colony isolates from one of the scale tip rot agar plates (Table 1, plate b), and the mixed colony from another (plate a) gave lesions; the other 5 isolates did not. Lesions began appearing on the wounded scales within 24 hours, and were definite after 48 hours. They continued to expand for a week or more. Some became confluent and destroyed whole scales; others were eventually limited and remained discrete. The results of re-isolations and some further inoculations are in Table 1.

The last 2 trials in Table 1 illustrate the early reactions of infected scales unwounded and planted in soil. The few that became obviously infected were not strictly unwounded; they had been broken from the bulbs 2–3 days before inoculation, and they rotted at the base where the break occurred. In Petri dishes, however, similar scales inoculated without wounding did not rot at the base. Some plants from this experiment and others from a larger one are being grown on. Preliminary results suggest that the scale tip rot bacterium may remain with the developing bulbil plant, and continue to act as a low-grade pathogen, attacking mainly the roots and sometimes the bases of outer scales on the small bulbs.

With one exception, only pathogen-free scales, bulbils, and small plants have been inoculated, and there is so far little evidence whether the bacterium alone will produce typical scale tip rot on larger bulbs. Scale tip rot symptoms have appeared on 2 bulbs of the few larger plants from the pathogen-free stocks that could be spared for inoculation.

Tentative identification of the scale tip rot bacterium.—Two representative cultures of the pathogenic bacterium (composing isolate *a* below) were submitted to Dr. M. P. Starr, University of California, Davis, for identification or description. Mrs. Gladys Cosens, of his laboratory, reported that standard tests show it to be a *Pseudomonas* sp., but the species has not yet been determined. A nonpathogenic bacterium from a scale tip rot lesion that was used in control series (isolate *b* below) was not a *Pseudomonas*.

Fungi isolated from scale-tip-rot-infected bulbs.—*Cylindrocarpon radicicola* and *Fusarium oxysporum*

Schlecht. were common on the underground parts of the Croft lily plants from which the scale tip rot bacterium was isolated. They were present in tissue, bearing lesions and surface damage, from bulb scales and basal plates and from roots. Samples of each species were taken through 1–3 single-spore transfers, and representative isolates were tested in pathogenicity trials. Two *C. radicicola* cultures and 1 *F. oxysporum*, used consistently for tests of interaction with the scale tip rot bacterium, were originally from lightly sterilized yellowed surface tissue of scales. Other isolates of the 2 species used once or twice came from scale tip rot lesions, and several other *F. oxysporum* isolates were from roots and basal bulb rot lesions.

Slight symptoms were produced by the *C. radicicola* and *F. oxysporum* isolates, wound-inoculated on pathogen-free scales. Though very slight, the symptoms were distinguishable from the normal reactions of the tissue to wounding. Two or 3 days after the scales were stabbed in the presence only of distilled water, an almost microscopic rim of callus formed around each small wound. Within this rim, dead and damaged cells turned light brown (Fig. 5-h). Both fungus species stopped this normal callus formation. *C. radicicola* produced a red spot or rimmed lesion around each wound (Fig. 5-b,d), and *F. oxysporum* slightly extended the damaged area but caused little or no change in color (Fig. 5-f). Each fungus was consistently and almost exclusively re-isolated in pure culture from surface-sterilized tissue from around the wounds into which it had been inoculated.

C. radicicola sometimes penetrated unwounded small scales or the centers of bulblets, and rotted them. The color of the rotting tissue was reddish brown. On older unwounded scales, it sometimes caused almost microscopic superficial lesions by killing small groups of epidermal cells. Evidence was seen that the *F. oxysporum* isolate slightly extended the volume of dead tissue at the tips of unwounded scales (4).

Mixed inoculations.—The interactions between bacteria and fungi isolated from lily scales were tested in 7 trials, 4 of which are discussed below. Two were designated combination trials, because each of 3 bacterial isolates was paired with each of 3 fungi, before inoculation to pathogen-free lily scales. In addition, two dilution trials tested the consequences of mixing the scale tip rot *Pseudomonas* at 3 dilutions with an isolate of *C. radicicola* at 3 dilutions before inoculation.

The isolates used in the combination trials were as follows:

a) A 2-day culture of the scale tip rot bacterium (*Pseudomonas* sp.) in beef-peptone broth was diluted 1:20 with distilled water. Two single-colony isolates were mixed in the inoculum. Both were consistently pathogenic but were known to differ in the development and zonation of lesions (results of 5 trials).

b) A bacterium of another species that caused slight yellowing of bulb scales but no rot. Isolated from a scale tip rot lesion.

c) A nonpathogenic bacterium isolated from check scales.

d) A distilled water blank.

A) A suspension in distilled water of spores and fragmented hyphae of a single-spore isolate of *C. radicicola*, capable of producing very slight symptoms on wounded bulb scales.

B) A single-spore isolate of *C. radicicola* producing definite small red lesions on wounded scales.

C) A single-spore isolate of *F. oxysporum* very slightly pathogenic on wounded scales.

D) A distilled-water blank.

All 4 of the bacterial series were paired with all 4 of the fungal series. Mixtures were made before inoculation. The second experiment was a repetition of the first, and the results are discussed together. Altogether, 12 scales per treatment were used in the 2 trials. Six were pricked, each 10 times, with a sharpened wooden applicator stick, and 6 were unwounded.

The 2 dilution trials were made with the scale tip rot *Pseudomonas*, isolate *a*, and *C. radicicola*, isolate *B*, that produced definite lesions. The results of the dilution trials, being dependent on the concentration of organisms in the inoculum, differed quantitatively but revealed the same phenomenon.

Combination trials.—Two types of interaction were

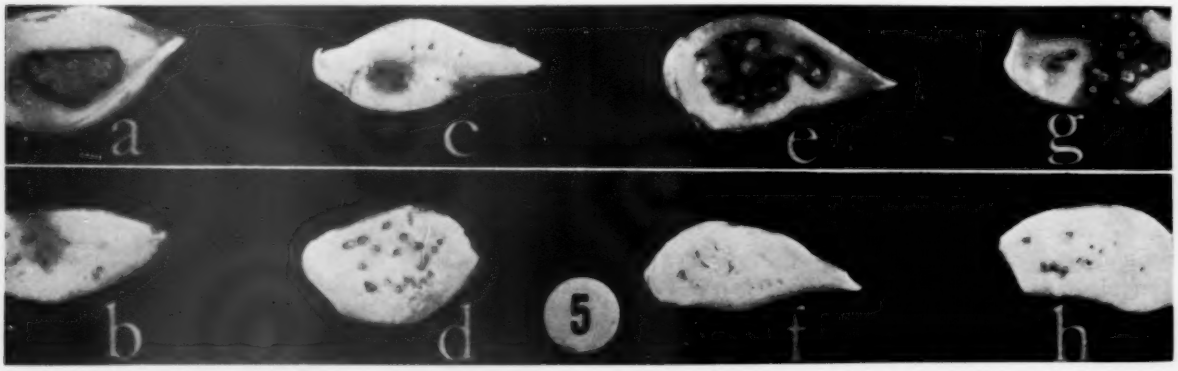

Fig. 5.—Wound-inoculated scales from 8 of 16 treatments in a combination trial. **a)** *Cylindrocarpon radicicola*, isolate *A* plus *Pseudomonas* sp. **b)** *Cylindrocarpon radicicola*, isolate *A* only.) **c)** *Cylindrocarpon radicicola*, isolate *B* plus *Pseudomonas* sp. **d)** *Cylindrocarpon radicicola*, isolate *B* only. **e)** *Fusarium oxysporum* plus *Pseudomonas* sp. **f)** Fusarium *oxysporum* only. **g)** *Pseudomonas* sp. only. **h)** Distilled water blank.

Table 2.—Dilution and inoculation of mixed and single organisms, *Pseudomonas* sp. and *Cylindrocarpon radicicola*. Mean lesion diameter in mm.

Pseudomonas isolate	Dilution by:	Cylindrocarpon isolate B 1	B 10	B 100	Water (D)
a	1	6.1	5.5	6.1	6.3
a	10	3.3	3.8	5.3	5.7
a	100	3.3	4.4	1.8	5.2
Water (d)		3.5	3.3	3.2	2.5

Least significant difference, 1.3.

observed between the scale tip rot bacterium and fungi. Mixing *Pseudomonas* and *C. radicicola*, isolate *B*, suppressed the characteristic symptoms of both organisms, i.e., of the rotting caused by Pseudomonas alone, and the small red lesions caused by *C. radicicola* alone. The contrast appears in Fig. 5-c from the mixed inoculation, and Fig. 5-g,d from the single inoculations.

The second type of interaction appeared when the *Pseudomonas* was inoculated in a mixture with *F. oxysporum*. At first the symptoms were similar to those of the bacterium alone (Fig. 5, compare e and g); later *F. oxysporum* expanded the lesions, destroying whole scales, and produced sporulating mycelium over the rotten tissue (Fig. 4).

Apart from these 2 mixtures, evidence of interaction was slight or absent. The *C. radicicola* isolate, *A*, which caused barely evident lesions (Fig. 5-b), slightly reduced the expansion of the bacterial lesions (Fig. 5-a), but it did not greatly alter their appearance (Fig. 5-g).

The difference between the mild and the severer isolates of *C. radicicola* in their antagonism to the scale tip rot bacterium may have been associated with the greater capacity of *B* to become established in the bulb scale tissue. Evidence for different degrees of penetration appeared in re-isolations from inoculated tissues: for example, in the first of the 2 combination experiments re-isolations from 11 *d* + *A* tissue pieces gave 5 colonies of *C. radicicola*, whereas 6 pieces remained sterile. All of 16 tissue pieces from series *d* + *B* gave *C. radicicola*. No attempt was made to re-isolate from tissues inoculated with mixtures of organisms.

In other mixtures, the nonpathogenic bacterium *b* caused a slight increase of discoloration in the small superficial lesions caused by the 2 *C. radicicola* isolates and *F. oxysporum*. The bacterium, c, had no influence on the slight lesions produced by the fungi.

Dilution trials.—In the 2 dilution trials, the organisms were combined in the 16 mixtures and single suspensions, as shown in Table 2. To obtain the "undiluted" *Pseudomonas* inoculum for the first trial, a very turbid 4-day broth culture was diluted 1:20. The inoculum of *C. radicicola* was made with the spores and fragmented mycelium from a single heavily sporing PDA slant suspended in 40 ml water. In the second trial less concentrated inocula were prepared from a 2-day broth culture of *Pseudomonas* diluted 1:50, and from the spores and mycelium on 2 slants of *C. radicicola* suspended in 200 ml distilled water.

In the first dilution trial the proportions of the 2 organisms successfully included the range of antagonistic action. Visible interaction of the fungus and bacterium, where it occurred, was both quantitative and qualitative—a change in size of lesions, and a change in the type of lesions produced. The appearance of lesions was recorded on color film and, from the film, their diameters were measured and recorded as mean size in mm of a single lesion (Table 2).

All dilutions of the single inocula gave characteristic symptoms. In the set of combinations *a*1 + *B*1, *a*10 + *B*10, *a*100 + *B*100 (Table 2), in which the organisms were in the same relative proportions but at different concentrations, bacterial lesions developed rapidly and severely on the scales inoculated from the highest concentration *a*1 + *B*1; they developed but were restricted in the *a*10 + *B*10 series; and they were completely absent from the most dilute, the *a*100 + *B*100 series. The degree of suppression of the lesions from other combinations was symmetrical about this set of treatments: where there were relatively higher concentrations of *Cylindrocarpon* than *Pseudomonas* the lesions were reduced; where *Pseudomonas* predominated, the lesions were normal. The contrast is made clear by comparing the lower left and upper right sections of Table 2.

Qualitatively, *Cylindrocarpon* in the mixtures produced lesions with a red rim and tended to redden rotted tissues. Concentrated inoculum of the bacterium alone gave light-colored lesions that later might have a darker rim; lesions from dilute inoculum often had a darker-colored rim earlier in their development. The scales inoculated with balanced dilute inoculum, *a*100 + *B*100, were in striking contrast to the scales with red lesions of more concentrated mixtures—there was no noticeable discoloration around the wounds, and the wounds closed; whereas even on the water checks, *d* + *D*, some of the wounds healed less completely because saprophytic bacteria multiplied in water drops above them.

Throughout the series inoculated with the highest concentration of *Pseudomonas* the unwounded scales showed much patchy surface discoloration, yellowish where there was no *Cylindrocarpon* (*a*1 + *D*), red at the highest concentration of *Cylindrocarpon* (*a*1 + *B*1). Where the tips of scales had died back before they were inoculated, normal on many scales, bacteria alone gave them a yellow-brown color with a line of denser color at the edge of the living tissue. *Cylindrocarpon* reddened these dead scale-tip tissues.

The second dilution trial was designed to include a lower range of inoculum concentrations, but the proportions of the two organisms most likely to result in antagonism apparently lay between those of the prepared inocula. The combination showing most evidence of antagonism was *a*10 + *B*1 (Table 2) but the degree of suppression was hardly greater than in the *a*10 + *B*10 mixture of the previous trial. The more dilute combination, *a*100 + *B*10, in the same pro-

portions was free of symptoms; the symptoms from the dilute bacterial inoculum alone, *a*100 + *D*, were slight though definite. Since 2 other combinations grouped around *a*10 + *B*1, i.e. *a*10 + *B*10 and *A*100 + *B*1, also gave some evidence of symptom suppression, there is no doubt of the over-all effect. Thus, the same general phenomenon emerged in the second dilution trial as in the first, though not so clearly.

Discussion.—There is not yet full proof that a *Pseudomonas* sp. produces the characteristic symptoms of scale tip rot on mature bulbs, thus causing the disease as known in the field. It is likely to be 1–2 years before evidence is sufficient from experiments under controlled conditions. In the meantime it is reasonable to assume that the *Pseudomonas* sp. is the primary pathogen of scale tip rot. Its pathogenicity on lily scales has been proved consistently in a number of trials. Also, recent findings indicate it is far from being relatively harmless, merely disfiguring infected bulbs. Underground stem lesions on Croft lilies, long attributed to physiological causes, have yielded bacteria culturally similar to the scale tip rot *Pseudomonas*. The stem lesions are often associated with a serious reduction in the vigor of field-grown Croft lily plants. In addition, under greenhouse conditions, the severity of scale tip rot on bulbs planted in greenhouses for forcing has been associated with the premature shriveling of lower leaves and, to a lesser degree, leaf scorch. Such associations exist among plants free of serious symptoms of root rot.

Evidence of antagonism between *C. radicicola* and the scale tip rot bacterium appeared in all of the 4 trials mentioned herein, and in 3 others. The antagonism seemed to be mutual, since the growth of both organisms was checked in one set of mixed cultures on agar. Inoculated together on lily scales, there were 1) a conspicuous effect, the suppression of bacterial rotting, and 2) a less noticeable effect, the suppression of the reddish discoloration characteristic of the small *S. radicicola* lesions. There is no direct evidence on the nature of this mutual inhibition, but the suggestion already given, that inhibition occurs during growth in the same medium, receives support from the dilution trials. If it were due to the lethal action of toxins present in cultures or suspensions of the 2 organisms, mixing them before inoculation should have been most lethal at high concentrations of inoculum. The phenomenon of antagonism appeared mainly at lower concentrations. The action of the 2 organisms on each other seems to have been static rather than lethal.

An example of antagonism somewhat like that between the *Pseudomonas* sp. and *C. radicicola* has been described by the senior author (1). *Rhizoctonia solani* Kühn and *Streptomyces scabies* Thax. were distributed on the surfaces of potato tubers in a way that suggested mutual antagonism. The distribution was such that it gave a negative association between the incidence of scab lesions and Rhizoctonia sclerotia on single tubers. There were also indications that elements of the microflora present on a potato tuber come from the parent tuber by way of the developing shoot and stolon; other elements are derived from the soil. The microflora of lily bulbs—pathogenic, synergistic, competitive, antibiotic and neutral—probably have a similar dual source. The suggestion made for the potato diseases (1), of working toward control methods by employing a simultaneous application of pesticides and antagonists to the planting material, is still far in the future. For lily bulbs, the more direct approach, using pesticides with a spectrum covering the range of known pathogens, gives a chance of immediate success. Since the discovery of a bacterial pathogen on lily bulbs, trials are being made of a bactericide, Agrimycin, incorporated in the PCNB-ferbam dip (3) now commonly used by bulb growers. Results are not yet available.

The mutual antagonism between the scale tip rot bacterium and *C. radicicola* and the synergistic effect of *F. oxysporum* suggest explanations for common inconsistencies in results from previous pesticidal treatments of bulb and soil. Together they compose a model system likely to react in any of 3 ways to treatment with a pesticide. The lethal effect might be on *C. radicicola*, removing its antagonistic action and making scale tip rot worse. The pesticide might check scale tip rot directly by action on the bacterium, or indirectly by action on the *Fusarium*. By different degrees of action on 2 or 3 organisms it might worsen the disease, leave it much the same, or reduce it. Such explanations have often been given for erratic results from the application of pesticides, but few model systems to which they might apply have so far been available for controlled experiments.—Department of Plant Pathology, University of California, Los Angeles.

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PEA INTRODUCTIONS WITH PARTIAL RESISTANCE TO APHANOMYCES ROOT ROT

John L. Lockwood

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Cooperation of the U.S.D.A. Research Service, Crops Research Division, New Crops Research Branch; W. H. Skrdla; and the North Central Regional Plant Introduction Station, Ames, Iowa, in making pea introductions available is acknowledged. Appreciation is expressed to Richard A. Crum for technical assistance; to Ferry-Morse Co., Detroit, Mich., for supplying seed of Miragreen peas; to W. T. Schroeder and D. W. Barton, New York Agr. Exp. Sta., Geneva, for supplying a seed sample of P. I. 169604 selection and isolate 72 of *Aphanomyces euteiches;* and to L. O. Lawyer, California Packing Co., San Leandro, for supplying isolates 102 and 103 of *A. euteiches.*

SUMMARY

Several pea introductions gave consistently lower disease indices than commercial varieties in replicated tests in the greenhouse in sand. Those with lowest disease indices were P.I. numbers 166159, 167250, a selection of 169604, 180693, 180702, 180868, and 184129. Those with highest levels of resistance had disease indices of about 3.0, compared with disease indices of about 8.0 (the maximum disease index, indicating dead plants) for commercial varieties. Significant correlation coefficients existed for disease indices of P.I. 169604 selection and Miragreen, and of P.I. 180693 and Miragreen. Several accessions consistently gave resistant reactions when tested under variable conditions in the greenhouse. Variable factors were a) zoospore concentration, b) age at which plants were inoculated, c) temperature of sand, d) isolate of *Aphanomyces euteiches*, e) age of culture. P. I. 180693 and P.I. 169604 selection were less severely diseased than Miragreen when grown in the greenhouse in steamed soil infested with the pathogen. One of the 4 isolates of *A. euteiches*, isolate 102, appeared to be pathogenically distinct from 3 other isolates.

During an extensive greenhouse screening program for evaluating pea introductions for resistance to root rots caused by *Aphanomyces euteiches* Drechs. and *Fusarium solani* (Mart.) App. & Wr. f. *pisi* (F. R. Jones) Snyd. & Hans., several accessions with slight resistance to *A. euteiches* were found (unpublished data). Selected introductions were studied further in an attempt to determine whether or not their resistance was of a sufficiently high level and expressed over a sufficiently wide range of conditions to suggest promise in a program of breeding for root rot resistance. The response of the variety Miragreen to some conditions affecting Aphanomyces root rot was studied previously (1).

Materials and methods.—The procedure for producing Aphanomyces root rot in the greenhouse and for evaluation of disease resistance was described previously (1). For experiments in sand, 4 rows (replications) of 15 seeds per row were used. The variety Miragreen was used as the susceptible control. Each row was inoculated with 10 ml of a suspension containing 5×10^4–1.5×10^5 zoospores per ml, except where noted. Temperature tanks at 24°C were used. Disease index was the sum of the disease ratings of foliage (maximum, 3), epicotyls (maximum, 2) and roots (maximum, 3) of plants. Ratings were made for each row and averaged. Four isolates of *A. euteiches*, numbers 50, 72, 102, and 103, were used.

For tests in soil, 6-in. pots of steamed soil were planted to 8 pea seeds per pot. Soil was infested with *A. euteiches* 0, 8, and 21 days after planting by pouring 25 ml of inoculum into 2-in.-deep holes made in the soil. Inoculum was a 1-week-old Petri-dish culture on potato-dextrose agar (per liter: extract from 200 g potatoes, 20 g dextrose) mixed with 90 ml of water. Four pots were used for each treatment and planting was spaced so that all pots were inoculated the same day. Disease ratings for each pot were made 3 weeks after inoculation.

Analysis of variance was applied to the data.

Results.—*Comparison of selected pea introduction.*—Plant introductions that demonstrated resistance to Aphanomyces root rot in screening tests (unpublished data) were tested 4–18 times in replicated experiments to determine those with highest levels of resistance. Several accessions consistently showed higher levels of resistance than commercial varieties (Table 1). As an indication of the level of statistical significance, the LSD at the 5% level for the individual experiments ranged from 0.7–1.6, and averaged 0.9. Thus the LSD for the average values in Table 1 would be still lower. Even the most resistant accessions, P.I. numbers 166159 and 167250, expressed most of their resistance in the foliage; roots and epicotyls were always diseased, though to a somewhat lesser extent than in commercial varieties. The disease index of about 3.0 exhibited by these 2 accessions was the sum of the ratings 0–1–2, or 0–0.5–2.5 for foliage, epicotyls, and roots, respectively. Introductions with slightly higher indices generally had more severe root and epicotyl damage; those with still higher disease

Table 1.—Average disease indices of pea introductions infected with *Aphanomyces euteiches* in greenhouse tests.

P.I. no. or var.	Av. disease index[a]	P.I. no. or var.	Av. disease index[a]
166159	2.7	173059	5.7
167250	3.1	212916	5.8
180693	3.8	175232	6.0
169604 sel.	4.1	164669	6.3
184129	4.5	169604	6.9
180868	4.8	Miragreen	7.6
180702	5.2	Early Perfection	7.8

[a] Average disease index was based on 4–18 tests of 4 replications each. Disease index was based on a scale of 0–8, with 0 indicating healthy plants, and 8, dead plants. LSD at 5% level for individual tests was 0.7–1.6.

indices had wilted foliage. A selection of P.I. 169604 was significantly more resistant than the parent line in several tests (Table 1). A green, wrinkled-seeded selection from 169604 selection was more susceptible (disease index 5.5) than the predominantly smooth, purple-mottled-seeded 169604 selection (disease index 3.8) in one test. In the same test disease index for Miragreen was 8.0. These values differed statistically at the 1% level of significance.

The relation between disease reactions of P.I. numbers 166159, 169604 selection, and 180693, respectively, and Miragreen was studied by plotting data from numerous experiments graphically (Fig. 1–3). Each point represents the average of 4 replications. Respective regression coefficients for the 3 pairs of varieties were 0.1, 0.8, and 1.1. Respective correlation coefficients were 0.72 and 0.78 for P.I. 169604 selection-Miragreen and P.I. 180693-Miragreen. These values were statistically significant at the 1% level, and showed that the 2 pea introductions were consistently, though incompletely, resistant to Aphanomyces root rot. Generally speaking these accessions had disease indices 3–4 units lower than did Miragreen. The correlation coefficient for the pair P.I. 166159-Miragreen was 0.41. A coefficient of 0.51 is required for significance at the 5% level.

Zoospore concentration.—The effect of concentration of zoospores of *A. euteiches* in the inoculum suspension on the expression of resistance to root rot in pea introductions 180693, 180702, 184129, 212916, and 169604 selection was studied in 4 experiments. A range of zoospore concentrations of 1×10^4–2.5×10^5 per ml was used. Typical results are in Fig. 4. Although disease in all 6 varieties increased with inoculum concentration, the same relative degree of resistance was expressed by the plant introductions at each concentration. Thus resistance in pea introductions was expressed over a wide range of inoculum levels.

Age of seedlings.—Pea introductions 180693, 180702, 184129, 212916, 169604 selection, and Miragreen were tested to determine the expression of resistance in seedlings inoculated later than 4–5 days after planting, the usual inoculation time. No differences in resistance were found in 2 experiments when the plants were inoculated 4 and 8 days after planting. A similar experiment was done with P.I. numbers 180693 and 169604 selection and Miragreen in steamed soil (Fig. 5). Seedlings were inoculated 0, 8, and 21 days after planting. Disease in all 3 varieties tended to decrease with increasing age of the plants at inoculation, but a similar relative degree of resistance was expressed by the 2 plant introductions inoculated at the 3 ages. It thus appears that the resistance of pea introductions

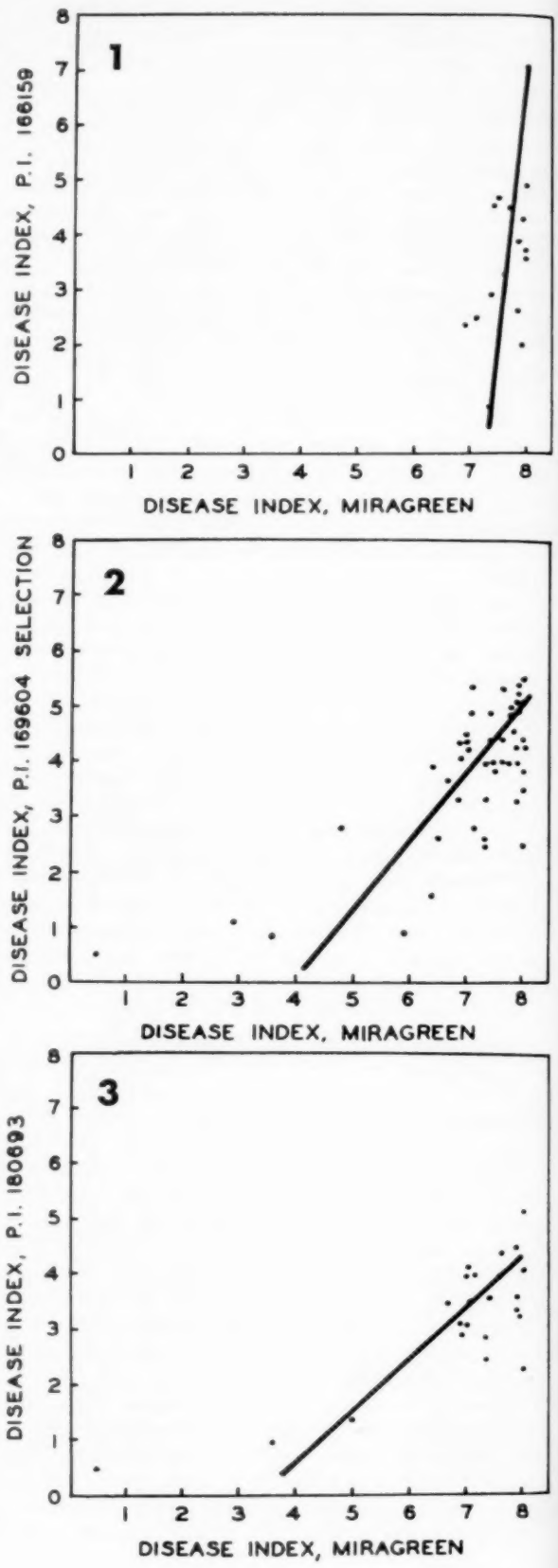


Fig. 1-3.—1) Relation between disease indices for P.I. 166159 and Miragreen. 2) Relation between disease indices for P.I. 169604 selection and Miragreen. 3) Relation between disease indices for P.I. 180693 and Miragreen. Disease index was based on a scale of 0–8, with 0 indicating healthy plants, and 8, dead plants. Each point is the average of 4 replications of 15 plants each for each variety.

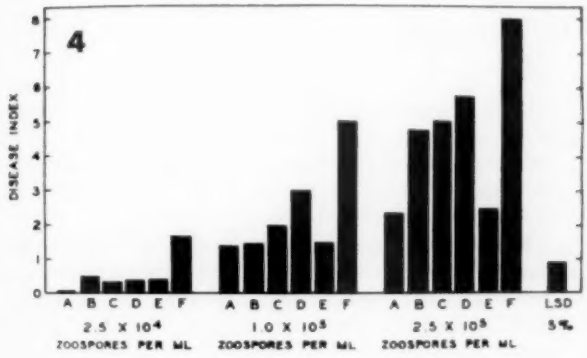
to Aphanomyces root rot is expressed over a range of ages, at least in plants inoculated up to 3 weeks old.

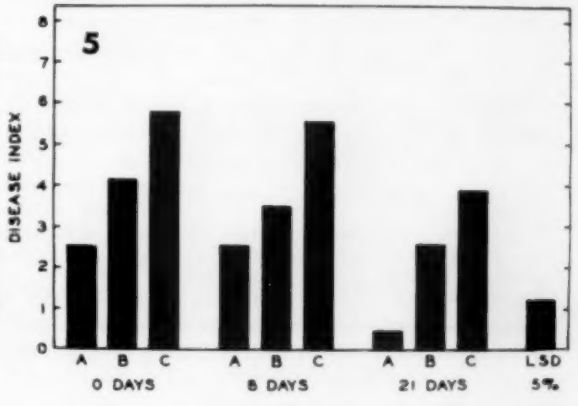
Temperature.—The resistance of pea introductions 180693 and 169604 selection to Aphanomyces root rot was tested at soil temperatures of 16, 20, 24, and 28° in 2 tests (Fig. 6). Disease indices for Miragreen increased with temperature up to 24°. Disease indices of P.I. numbers 180693 and 169604 selection increased with increasing temperature up to 28°. Differential resistance was expressed by the 2 pea introductions at all temperatures except 16° where disease index was 0.5 for all 3 varieties.

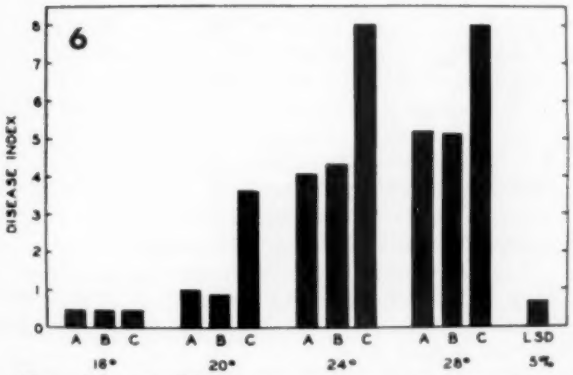
Isolates of A. euteiches.—Pea introductions 166159, 180693, 169604 selection, and the commercial varieties Miragreen and Early Perfection were inoculated with zoospores produced by 4 isolates of *A. euteiches* in 3 tests. All were inoculated with a concentration of 5×10^4 per ml. The respective disease indices expressed by the 5 pea varieties were similar whether inoculated with isolate 50, 72, or 103 (Fig. 7). Pea introduction 166159 was slightly susceptible, and 180693 and 169604 selection moderately so, whereas Miragreen and Early Perfection were severely diseased. A different pattern was obtained with isolate 102 which produced severe disease on Early Perfection, but was only slightly pathogenic on the other varieties. The results strongly suggest that isolate 102 differs in pathogenicity from the other 3 isolates. Possibly it is a distinct pathogenic race of the fungus.

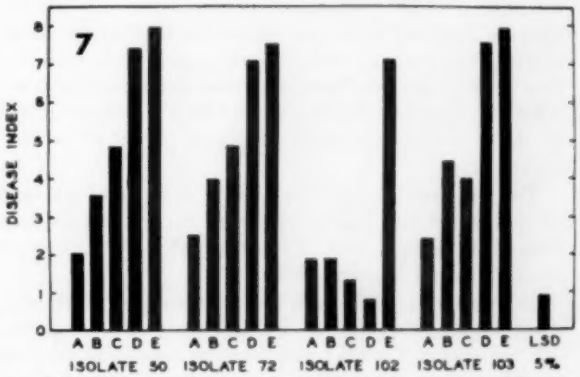
Age of culture.—The effect of age of the culture of *A. euteiches* on pathogenicity of zoospores to P.I. 169604 selection and Miragreen was studied in 3 experiments. In 2 of 3 tests seedlings of both varieties inoculated with the same concentration of zoospores from cultures 8 or 11 days old had significantly higher disease indices than plants inoculated with zoospores from cultures 4 days old (Table 2). In all 3 tests P.I. 169604 selection had significantly lower disease indices than did Miragreen, regardless of the age of the cultures.

Discussion.—The consistently lower disease indices expressed by certain pea introductions as compared with Miragreen, together with the fact that resistance in selected accessions was expressed over a wide range









Figs. 4-7.—**4)** Effect of concentration of zoospores of *A. euteiches* in the inoculum suspension of root rot in different pea varieties. **A)** P.I. 180693, **B)** P.I. 180702, **C)** P.I. 184129, **D)** P.I. 212916, **E)** P.I. 169604 selection, **F)** Miragreen. Least difference for statistical significance at 5% level was 0.9. **5)** Effect of age of plants at inoculation on resistance of pea varieties to Aphanomyces root rot in steamed soil in the greenhouse. **A)** P.I. 180693, **B)** P.I. 169604 selection, **C)** Miragreen. Least difference for statistical significance at 5% level was 1.2. **6)** Effect of temperature on expression of resistance in pea varieties to Aphanomyces root rot in sand. **A)** P.I. 180693, **B)** P.I. 169604 selection, **C)** Miragreen. Least difference for statistical significance at 5% level was 0.7. **7)** Pathogenicity of different isolates of *A. euteiches* to pea varieties. **A)** P.I. 166159, **B)** P.I. 180693, **C)** P.I. 169604 selection, **D)** Miragreen, **E)** Early Perfection. Least difference for statistical significance at 5% level was 0.9. Disease index was based on a scale of 0–8, with 0 indicating healthy plants, and 8, dead plants.

Table 2.—Pathogenicity of zoospores of *Aphanomyces euteiches* from cultures of different ages to P.I. 169604 selection and Miragreen peas.

Test	Age of culture	Disease index[a]		LSD[b]
		P.I. 169604 sel.	Miragreen	
1	4 days	6.5	7.6	0.6
	8 days	2.6	5.3	
2	4 days	4.0	7.5	1.0
	8 days	3.3	6.8	
3	4 days	4.6	7.8	1.6
	7 days	1.6	6.4	
	11 days	0.9	5.9	

[a] Disease index was based on a scale of 0–8, with 0 indicating healthy plants, and 8, dead plants.

[b] LSD (5% level) values apply both to age of culture and to varieties of pea.

of variable conditions, suggests that plant introductions may provide a suitable source of resistance for breeding peas resistant to Aphanomyces root rot. The expression of resistance by 2 pea introductions in steamed soil infested with the pathogen in the greenhouse also supports this view. Breeding for Aphanomyces resistance, however, may be complicated by the existence of pathogenic races of the fungus, as suggested by the present study. Furthermore, to obtain effective resistance to pea root rot, resistance to Aphanomyces root rot will undoubtedly have to be combined with resistance to *F. solani* f. *pisi*, another pea root pathogen common in Michigan pea fields (2).

Department of Botany and Plant Pathology, Michigan State University, East Lansing, Michigan.

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PHYTOPATHOLOGICAL NOTE

POLLEN TRANSMISSION OF NECROTIC RINGSPOT AND PRUNE DWARF VIRUSES IN SOUR CHERRY

R. M. Gilmer and R. D. Way

Respectively, Professor of Plant Pathology and Associate Professor of Pomology, New York State Agricultural Experiment Station, Geneva, New York.

Approved by the Director as Journal Paper No. 1203.

Way and Gilmer (3) presented limited evidence that necrotic ringspot virus (NRSV) was transmitted by pollen to seeds produced on healthy sour cherry trees (*Prunus cerasus* L.). Their evidence for pollen transmission of NRSV was not conclusive, for it did not exclude the possibility that the virus might have alternatively been transmitted by arthropods or nematodes. The present paper offers conclusive evidence that both NRSV and prune dwarf virus (PDV) are transmitted by infected pollen to seeds produced on virus-free sour cherry trees.

The 50 English Morello cherry trees used as seed (female) parents had been grown for 4 years in 8-in. pots in the greenhouse. Each tree differentiated about 25–30 blossom buds in 1958.

In February, 1959, each tree was indexed while dormant by bud-inoculating 4 Nanking cherry seedlings (*P. tomentosa* Thunb.) and 2 peach seedlings (*P. persica* Batsch.). Each tree was further indexed as it leafed out by mechanically inoculating cucumber seedlings (var. National Pickling) with juice extracts from its foliage. A final indexing, by bud-inoculation from each tree to Shirofugen flowering cherry (*P. serrulata* Lindl.), was performed in early June. In all indexing tests, each of the English Morello seed parents was found free of NRSV and PDV.

The seed parents were grouped into 4 lots containing about equal numbers of trees, and each lot was hand-pollinated with pollen from a different Montmorency sour cherry tree. To prevent self-pollination, the English morello blossoms were emasculated while in the "balloon" stage.

Montmorency pollen donors had been indexed previously in 1957 and 1958. Three of the donors (CR 3-25; CR 6-29; 15-1-2) were infected with NRSV and PDV; the remaining donor (Is-5) was virus-free. Large branches were removed from each donor tree in late February and maintained in the greenhouse until anthesis with their bases immersed in water. Whole anthers were removed from the flowers and stored in Petri dishes until dehiscence. Mechanical inoculations of cucumber and squash (var. Butternut) seedlings with triturated pollen demonstrated that pollen from the 3 infected donor trees was infected with NRSV (virus G) or PDV (virus B), confirming a report of Ehlers and Moore (1). (Identification of virus B with PDV is made by R. W. Fulton in a paper currently in press.) No virus transmission was obtained from pollen of donor Is-5, the virus-free control.

Fruits resulting from pollinations were harvested on May 18, 61–69 days after pollination. Seeds were after-ripened for 143 days at 40°F in polyethylene and planted in peat moss.

When seedlings reached the 2-leaf stage, small amounts of leaf tissue were excised from each seedling and triturated in 2–3 drops of potassium phosphate buffer (.05*M*, pH 7.6). The resultant juice extracts were each mechanically inoculated on the cotyledons of 4 cucumber seedlings. Each cherry seedling was thus indexed at least once, and, when possible, 3 times. Because of the presence of a few albino seedlings and

Table 1.—Virus transmission in seeds of healthy English Morello pollinated by 4 different Montmorency pollen donors.

	Pollen donors			
	Is-5	15-1-2	CR 6-29	CR 3-25
Virus status of pollen donor	Healthy	PDV+ NRSV	PDV+ NRSV	PDV+ NRSV
Seeds set/flowers pollinated	59/324	33/347	38/326	75/434
Seeds germinated	45	21	30	37
Seedlings infected with:				
PDV	0	7	2	1
NRSV	0	0	1	7
PDV+NRSV	0	1	1	2
Total infected	0	8	4	10

the operation of an apparent lethal gene, 27% of the seedlings died shortly after developing to the 2-leaf stage. Many of these seedlings could thus be indexed only once. At least 1 of the albino seedlings was virus-infected.

Table 1 records virus recovery from the seedlings. NRSV (virus G)and PDV (virus B) were identified by sub-inoculations from infected cucumber seedlings to Butternut squash and watermelon (var. Dixie Queen) (2).

These data demonstrate transmission of both NRSV and PDV by pollen to seeds produced on healthy English Morello trees. Thorough indexing of the seed parents—during dormancy, again in the first stages of active growth, and finally after the fruits were harvested—demonstrated the absence of both viruses in the seed parents. Germination of the seeds in peat moss and immediate indexing of the seedlings after germination precluded the possibility of virus transmission by nematodes or arthropods.

The major implications of pollen transmission of NRSV have already been pointed out (3), but one of them deserves brief repetition. The data presented show that about 25% of seeds resulting from fertilization of healthy ovules with pollen from diseased trees are infected with NRSV, PDV, or both viruses. Absence of NRSV and PDV from seed source trees thus affords no certainty that the seeds produced on such trees are virus-free.—New York State Agricultural Experiment Station, Geneva.

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A CALCIUM REQUIREMENT FOR GROWTH-REGULATOR-INDUCED RESISTANCE TO FUSARIUM WILT OF TOMATO.

Malcolm E. Corden and L. V. Edgington

Present address Botany and Plant Pathology Department, Oregon State College, Corvallis, Oregon.

The ability of plant-growth regulators to induce resistance to Fusarium wilt of tomato has been correlated by Corden and Dimond (2) with ability to inhibit root elongation. They suggest that these plant responses to growth regulators are related physiologically through increased calcium bonding of the pectic substances of the plant cell walls. The calcium pectates thus formed decrease cell wall plasticity (i.e., inhibit root elongation) (1), and are relatively resistant to hydrolysis by the fungal pectic enzymes (4), which have been implicated in the wilt disease syndrome (5). Edgington and Walker (3) have shown that calcium-deficient tomato plants are considerably more susceptible to Fusarium wilt than normal plants, and that plants receiving excess calcium are slightly resistant. They postulated that lack of calcium-bonded pectic substances facilitate increased degradation by fungal enzymes. The therapeutic activity of growth regulators should therefore be influenced by the amount of calcium available for reaction with the pectic substances of the plant cell walls. This note reports experiments designed to determine the interaction between growth-regulator treatments and calcium nutrition on Fusarium wilt development in tomato.

In a typical experiment 6-in. Bonny Best tomato plants were transplanted to Hoagland's solution with low calcium (20 ppm), normal calcium (200 ppm), and high calcium (1000 ppm). Half of the plants at each calcium level received α-naphthaleneacetic acid (NAA) at $10^{-6}M$ in the nutrient solution, and the other half were used as controls. The treatment solutions were changed on the 1st, 4th, and 6th days of treatment. On the 4th day NAA treatments were dis-

Table 1.—The effect of α-naphthaleneacetic acid (NAA) treatments and calcium nutrition on severity of Fusarium wilt of tomato as judged by vascular discoloration.

Treatment	Vascular discoloration index[a]	
	11 days after inoculation	22 days after inoculation
10^{-6} *M* NAA		
Low Ca (20 ppm)	0.62	
Normal Ca (200 ppm)	0.00	0.52
High Ca (1000 ppm)		0.30
No NAA		
Low Ca (20 ppm)	0.63	
Normal Ca (200 ppm)	0.16	0.97
High Ca (1000 ppm)		0.90
LSD (P = 0.05)	0.13	0.16

[a] Vascular index is the ratio of the number of large discolored bundles at all internodes to the total number of bundles observed (3 times the number of internodes per plant). The index ranges from 0, for a healthy plant, to 1, for a severely diseased plant.

Fig. 1.—Tomato plants treated with NAA and calcium and subsequently inoculated with *Fusarium oxysporum* f. *lycopersici*. **1)** Low calcium (20 ppm) and no NAA. **2)** Normal calcium (200 ppm) and no NAA. **3)** Normal calcium (200 ppm) and $10^{-6}M$ NAA. **4)** Low calcium (20 ppm) and $10^{-6}M$ NAA.

continued. On the 8th day the tap roots of the plants were severed in the distal region and inoculated by dipping the remaining roots in a suspension of washed bud cells of *Fusarium oxysporum* f. *lycopersici* (Sacc.) Snyd. & Hans. The plants were then placed in washed silica sand and watered periodically with the nutrient solutions they had been receiving before inoculation. Treatments were evaluated by the extent of vascular discoloration (Table 1, footnote a).

In plants rated for disease 11 days after inoculation (Table 1), treatment with NAA reduced vascular discoloration in those receiving normal calcium, but had no effect on plants receiving low calcium. Calcium-deficient plants were considerably more susceptible than plants receiving normal calcium. Foliar symptoms of the disease (Fig. 1) followed the same pattern as vascular discoloration following treatment with NAA and calcium.

In another set of plants rated 22 days after inoculation (Table 1), those that received NAA were considerably less diseased than those that received no NAA treatment. High calcium treatment caused a slight (statistically nonsignificant), decrease in disease in the plants with no NAA, but the plants receiving NAA and high calcium were significantly less diseased than those receiving normal calcium.

Thus, resistance to Fusarium wilt induced in tomato plants by growth regulator requires calcium and is enhanced by calcium levels above those necessary for normal plant growth. We might interpret these effects according to the hypotheses presented earlier (2, 3) as follows: Growth-regulator treatments allow demethylation of the plant pectic substances, but, without sufficient calcium present (i.e., low-calcium plants), rigid cross linkages fail to form and the pectic materials remain susceptible to fungal enzymes. When excess calcium is present (i.e., high-calcium plants), however, there is increased calcium bonding of the pectic substances, and this is particularly high when the number of bonding sites is increased by growth-regulator treatment.—Department of Botany and Plant Pathology, Connecticut Agricultural Experiment Station, New Haven.

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